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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2004902902 for a patent by BIOTRON LIMITED as filed on 31 May 2004.

WITNESS my hand this
Twelfth day of July 2004

J. Billingsley

JULIE BILLINGSLEY
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SUPPORT AND SALES

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AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

"ANTI-FLAVIVIRUS COMPOUNDS AND METHODS"

The invention is described in the following statement:-

ANTI-FLAVIVIRUS COMPOUNDS AND METHODS

FIELD OF THE INVENTION

The present invention relates to compounds and methods for retarding, reducing or otherwise inhibiting growth, or infection by, flaviviruses.

BACKGROUND OF THE INVENTION

The dengue virus belongs to the family of Flaviviridae and the genus Flavivirus. The Flaviviruses are a family of at least 66 viruses, 29 of which cause human diseases including dengue, yellow fever, Murray Valley encephalitis and Japanese encephalitis. As a well known and exemplary member of the Flaviridae family, dengue virus occurs mainly in tropical areas of Asia, Oceania, Africa, Australia and the Americas. Some outbreaks of dengue have involved one million or more cases with attack rates of 50-90%. Health experts have known about dengue fever for more than 200 years. The World Health Organization estimates 50 million cases of dengue infection occur each year which result in approximately 24,000 deaths (WHO 1998). This includes 100 to 200 cases reported annually to the U.S. Centers for Disease Control and Prevention (CDC), mostly in people who have recently travelled abroad. The global distribution of dengue is comparable to that of malaria and an estimated 2.5 billion people live in areas at risk for epidemic transmission.

Although not normally fatal, cases of haemorrhage and death have been described during outbreaks of classic dengue fever in Australia, Greece and Formosa. Dengue fever is an infectious disease carried by mosquitoes and caused by any of four related dengue viruses DEN-1, DEN-2, DEN-3, and DEN-4. A person can be infected by at least two, if not all four types at different times during a life span, but only once by the same type. People get dengue virus infections from the bite of an infected *Aedes* mosquito. Mosquitoes become infected when they bite infected humans, and later transmit the infection to other people they bite. The disease used to be called break-bone fever because it sometimes causes severe joint and muscle pain.

Most people recover from dengue fever completely within 2 weeks. Some, however, may go through several weeks of feeling tired and/or depressed. The acute phase, characterised by fever, myalgia, nausea, weakness and prostration, lasts about

7 days but there can be subsequent relapses. Majority of deaths that result from dengue infections are due to the development of severe bleeding problems - Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). The complication, dengue hemorrhagic fever, is a very serious illness which can lead to shock (very low blood pressure) and is sometimes fatal, especially in children and young adults. People who develop DHF have a 5% chance of death but if they go on to develop DSS then the mortality rate can rise as high as 40%.

Effective treatment for the dengue fever is unavailable. Prevention of epidemics relies mainly on attempts to eradicate the mosquito vector, *Aedes aegypti*. Vaccines have not proved successful because of the lack of protection across serotypes and the requirement of multivalent immunisation. Recently, however, attenuated candidate vaccine viruses have been developed in Thailand. These vaccines are safe and immunogenic when given in various formulations, including a quadrivalent vaccine for all four dengue virus serotypes. Efficacy trials in human volunteers have yet to be initiated. Research is also being conducted to develop second-generation recombinant vaccine viruses using the Thailand attenuated viruses as a template. Therefore, an effective dengue vaccine for public use will not be available for at least another 5 to 10 years.

Whilst ongoing research continues to provide further knowledge concerning the mode of action of Flaviviruses, including dengue, there is a pressing need for cure or prevention of the illnesses associated with this sub-group of viruses, particularly with the emergence of dengue virus as a growing global threat.

SUMMARY OF THE INVENTION

It has been surprisingly found that amiloride analogues or derivatives thereof are detrimental to the growth of the dengue virus and other Flaviviruses. In the context of the Flavivirus genus, and without being bound by any particular theory or mechanism of action, the negative effect of the amiloride analogues or derivatives appears to be exerted via the inhibition, or other negative effect, of the M protein of these viruses which are shown, for the first time in the present studies, to act as ion channels. As similar M proteins are present across the Flavivirus genus of viruses, the compositions

and methods of the present invention would have utility in the inhibition and/or treatment of infections by all Flaviviruses, including dengue virus.

The present invention is concerned with novel anti-flavivirus compounds comprising a guanidyl moiety and in particular to novel anti-flavivirus amiloride analogues and/or derivatives, novel anti-flavivirus cinnamoylguanidines or analogues and derivatives thereof, and novel anti-flavivirus napthoylguanidines, or analogues and derivatives thereof. However, it does not include in its scope the use of compounds 5-(N,N-hexamethylene)amiloride and 5-(N,N-dimethyl)-amiloride for retarding, reducing or otherwise inhibiting viral growth and/or functional activity of HIV.

Accordingly, a first aspect of the present invention provides a compound comprising a guanidyl moiety, having anti-flavirvirus activity.

According to a second aspect, the invention provides a compound selected from the group consisting of amiloride analogues or derivatives thereof, cinnamoylguanidines or analogues and derivatives thereof, and napthoylguanidines, or analogues and derivatives thereof, having anti-flavivirus activity.

According to a third aspect, the present invention provides a method of retarding, reducing or otherwise inhibiting growth of a flavivirus comprising contacting or exposing said virus to a compound selected from the group consisting of amiloride analogues or derivatives thereof, cinnamoylguanidines or analogues and derivatives thereof, and napthoylguanidines, or analogues and derivatives thereof.

According to a fourth aspect, the present invention provides a method of prophylactic or therapeutic treatment of a flavivirus infection comprising the administration to a subject requiring such treatment of a compound selected from the group consisting of amiloride analogues or derivatives thereof, cinnamoylguanidines or analogues and derivatives thereof, and napthoylguanidines, or analogues and derivatives thereof.

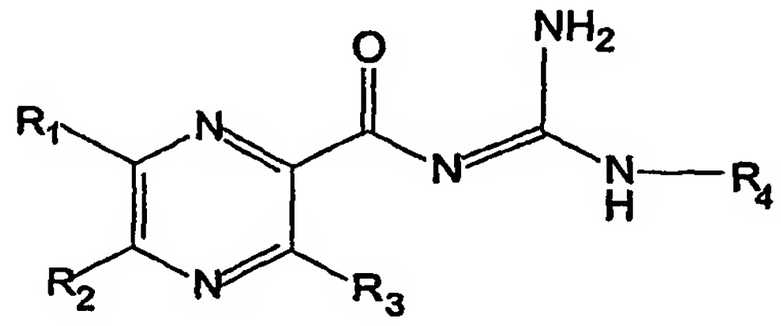
Preferably, the flavivirus to be inhibited or infection treated is that of the dengue virus.

Even more preferably, the flavivirus to be inhibited or infection treated is that of the dengue virus type 1 strain Singapore S275/90.

Other flaviviruses which can be inhibited or infections treated are those listed in Table 1.

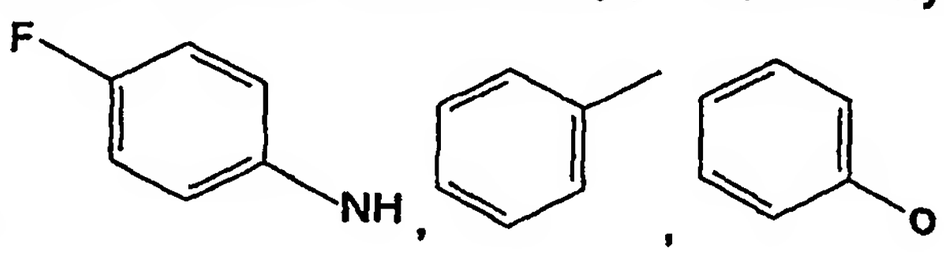
Examples of suitable compounds that can be used in the compositions and methods of the present invention are listed below.

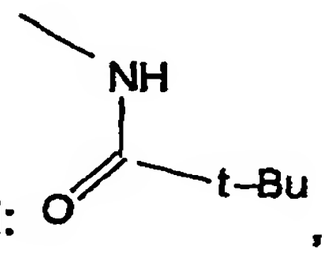
Amiloride analogues or derivatives comprising the structure:

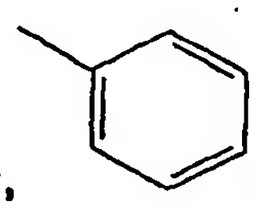
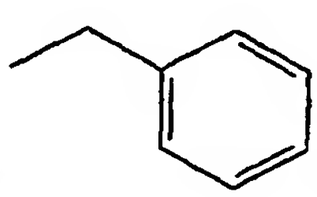
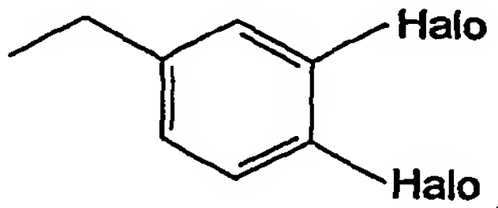


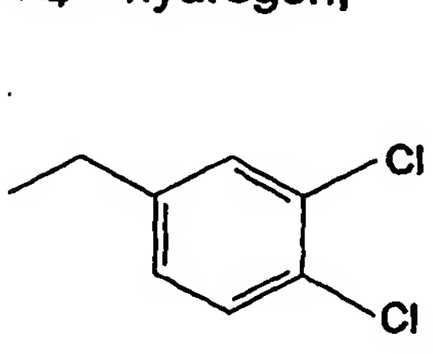
wherein the substituents at the R positions may or may not be the same, and

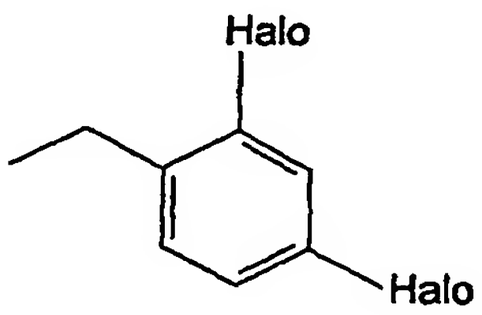
R_1 =hydrogen, halo, aryl, substituted aryl, phenyl, or substituted phenyl;

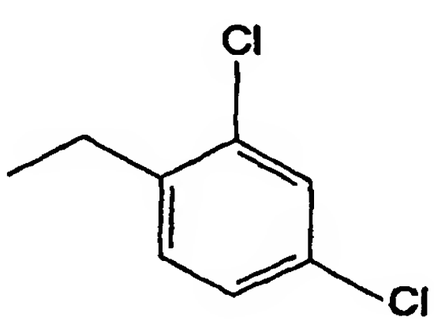
R_2 = hydrogen, amine, aryl, substituted aryl, halo, phenyl, substituted phenyl, hexamethylene, PrS, N-methyl-N-isobutyl, N-ethyl –N-isopropyl, benzyl ; N-methyl-N-guanidinocarbonyl-methyl, N,N-dimethyl, N,N-diethyl, tert-butylamino, halo-aniline, fluoro-aniline 

R_3 = hydrogen, hydroxy, halo, alkyloxy, methoxy, N,3-dimethylbutanamyl: 

R_4 = hydrogen,  or  or, 



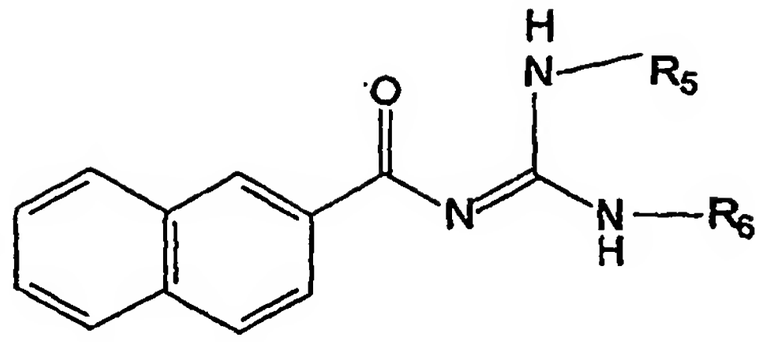
, 

, 

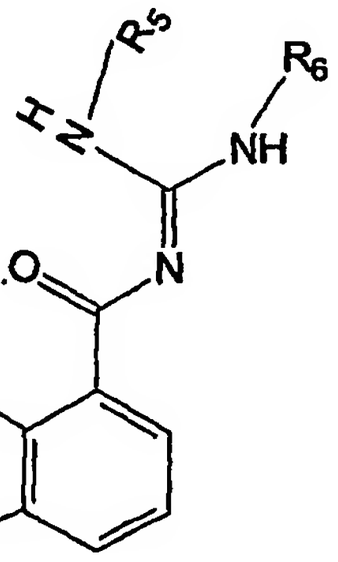
, or

— BOD^{IPY_FL};

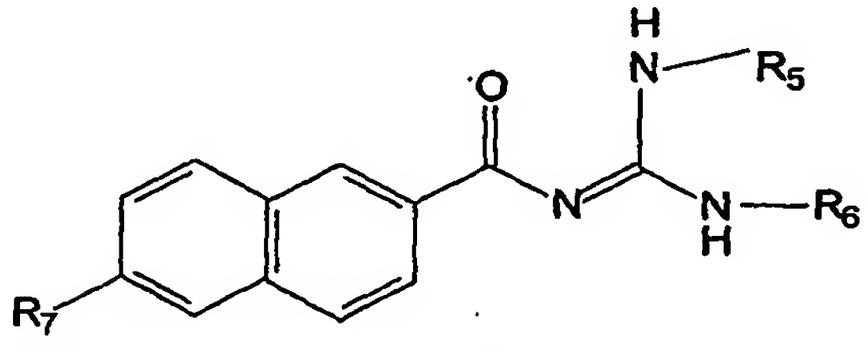
The following antiviral compounds comprising a guanidyl moiety are also encompassed within the scope of the present invention:



or



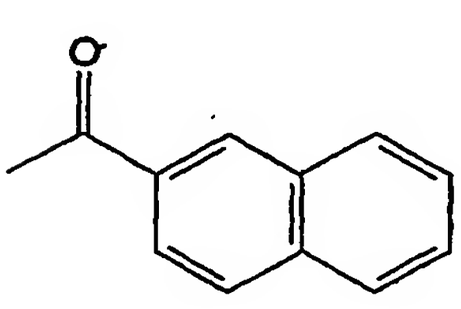
or



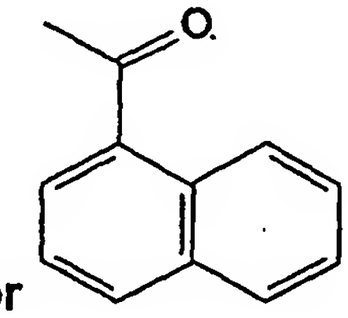
wherein the substitutents at the R positions may or may not be the same, and

R_5= Hydrogen, aryl, substituted aryl, phenyl, or substituted phenyl;

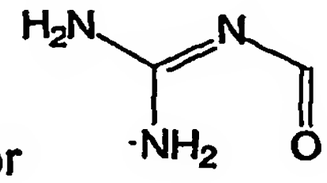
R_6 = Hydrogen, aryl, substituted aryl, phenyl, substituted phenyl, napthoyl,



, or

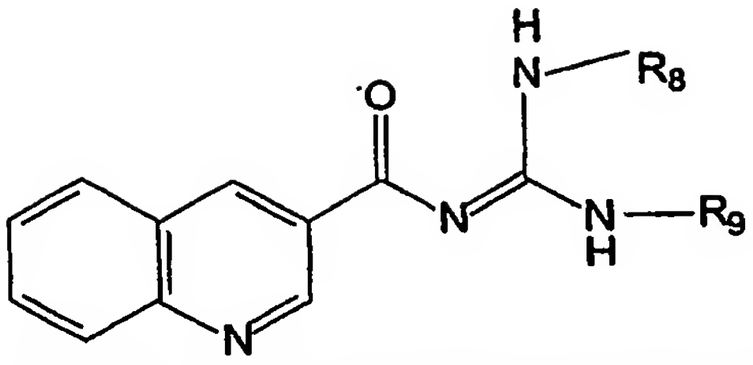


R_7 = hydrogen, halo, hydroxy , methoxy , alkyloxy , or



,

or, the structure

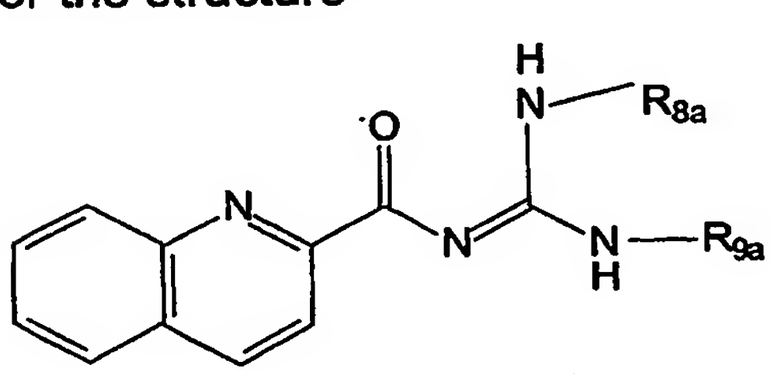


wherein the substitutents at the R positions may or may not be the same, and

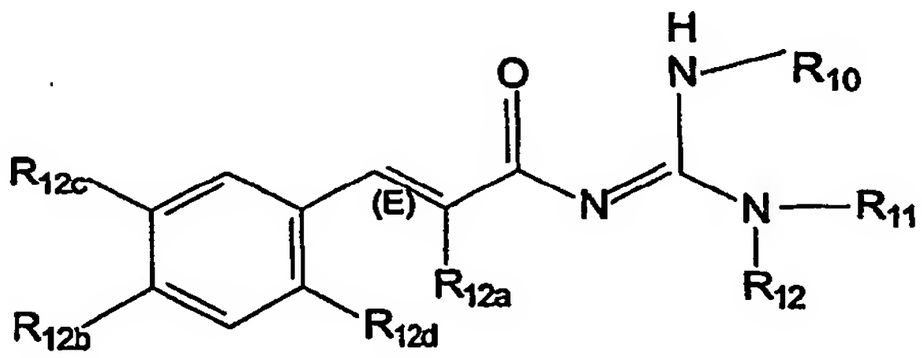
R_8 = aliphatic or aromatic substituents;

R_9 = aliphatic or aromatic substituents;

or the structure

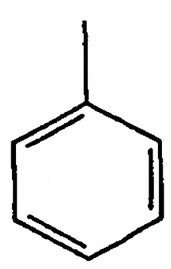
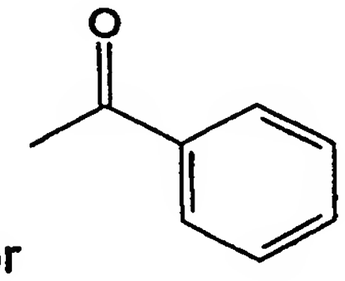


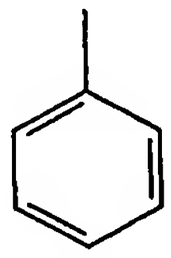
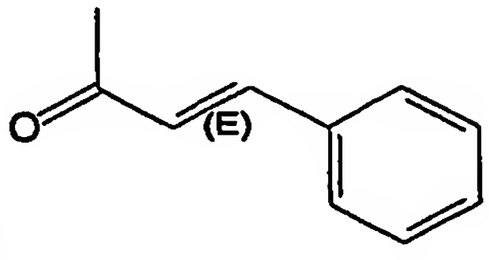
or, the structure



wherein the substitutents at the R positions may or may not be the same, and

R_{10} = hydrogen, aryl, phenyl, or cinnamoyl;

R_{11} = hydrogen , alkyl, aryl, phenyl, cinnamoyl;  , or 

R_{12} = hydrogen, alkyl, aryl, phenyl, cinnamoyl,  , or 

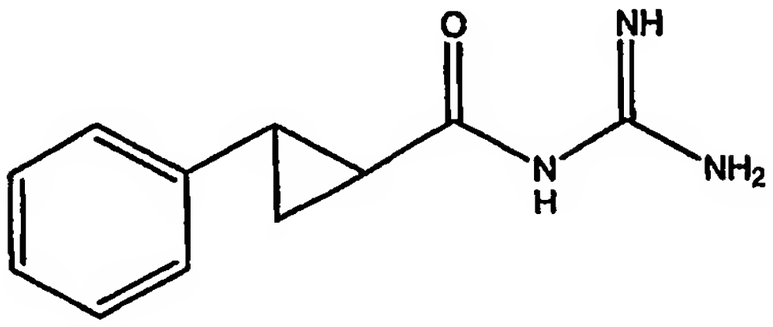
R_{12a} = hydrogen or alkyl,

R_{12b} = hydrogen, halo, hydroxy, alkoxy, or dialkyl amino,

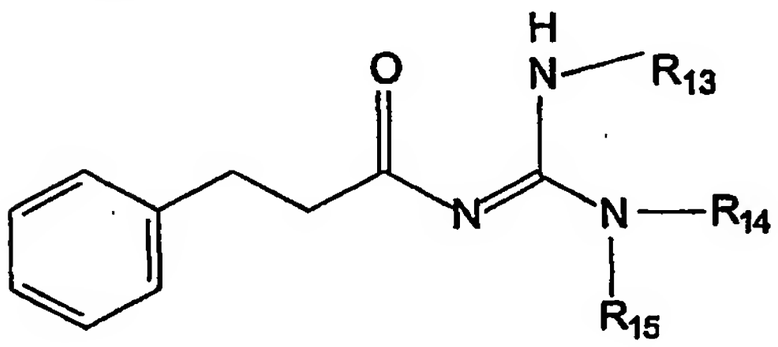
R_{12c} = hydrogen, halo or alkoxy,

R_{12d} = hydrogen, halo or alkoxy,

or the structure,

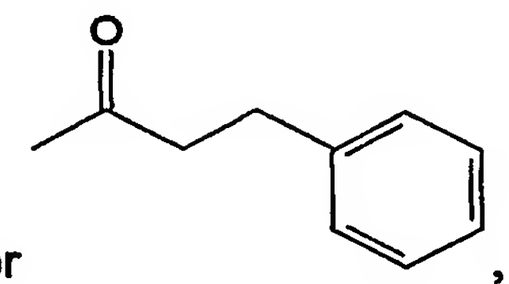


or, the structure



wherein the substitutents at the R positions may or may not be the same, and

R_{13} = H; alkyl, or phenyl

R_{14} = H, alkyl, phenyl, substituted phenyl, or 

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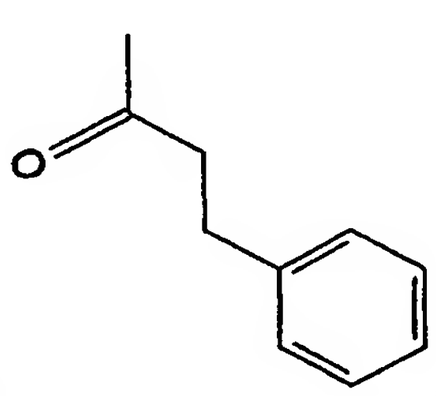
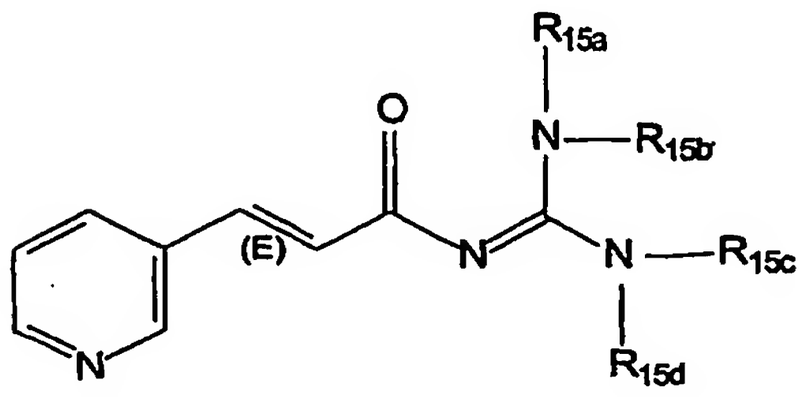


R_{15} = H, or ,

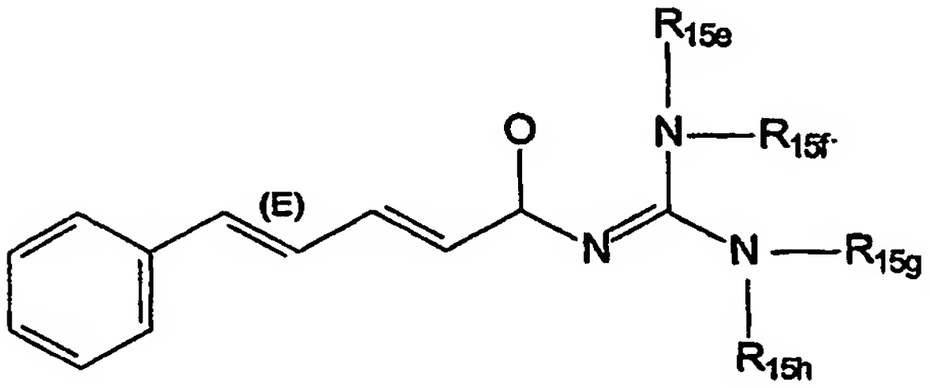
or the structure



wherein the substitutents at the R positions may or may not be the same, and

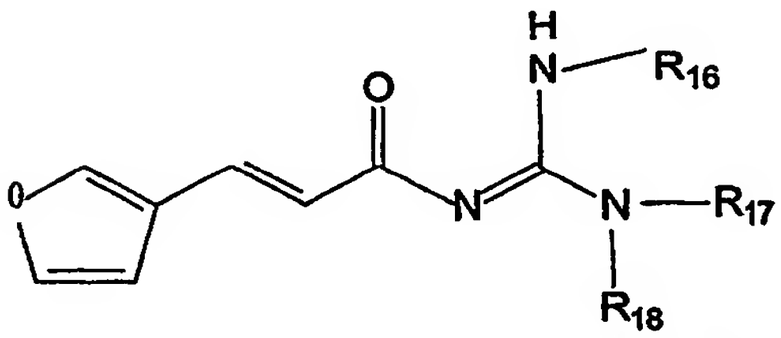
R_{15a}, R_{15b}, R_{15c} and R_{15d} = H; alkyl, or phenyl

or the structure



R_{15e}, R_{15f}, R_{15g} and R_{15h} = H; alkyl, or phenyl,

or the structure



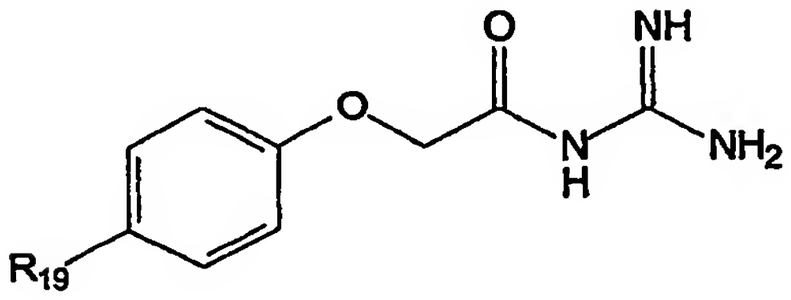
wherein the substitutents at the R positions may or may not be the same, and

R_{16} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

R_{17} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

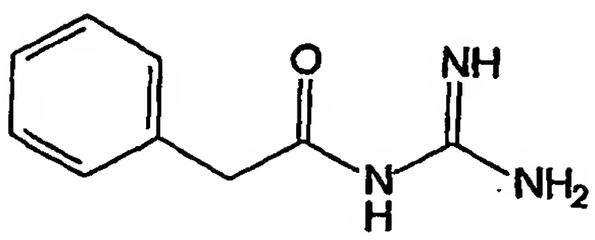
R_{18} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

or the structure



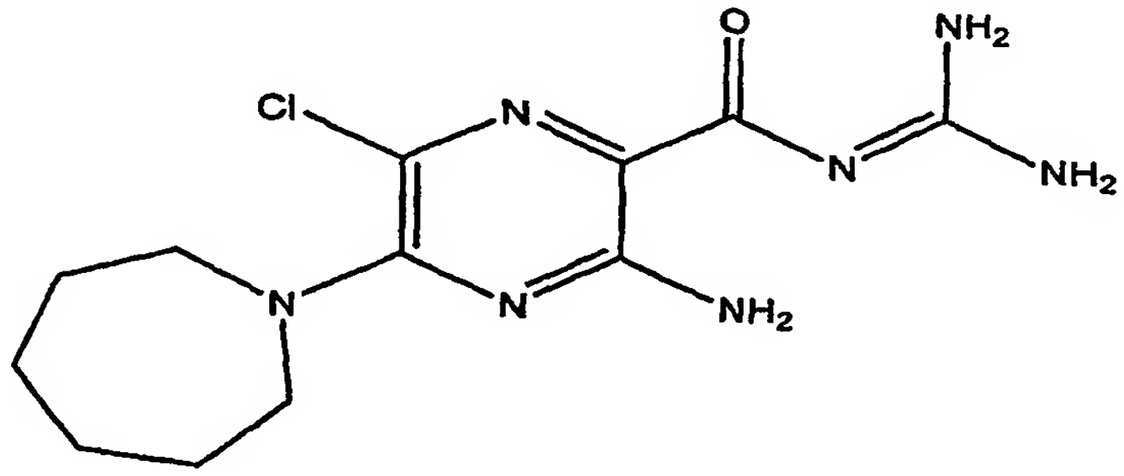
wherein R_{19} = hydrogen, halo; alkyl, hydroxy, alkoxy,

or the structure

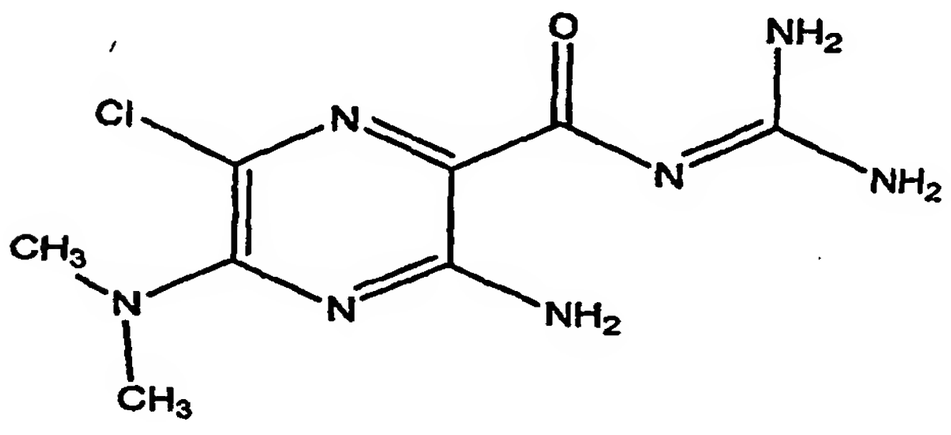


The compounds of the invention include the following:

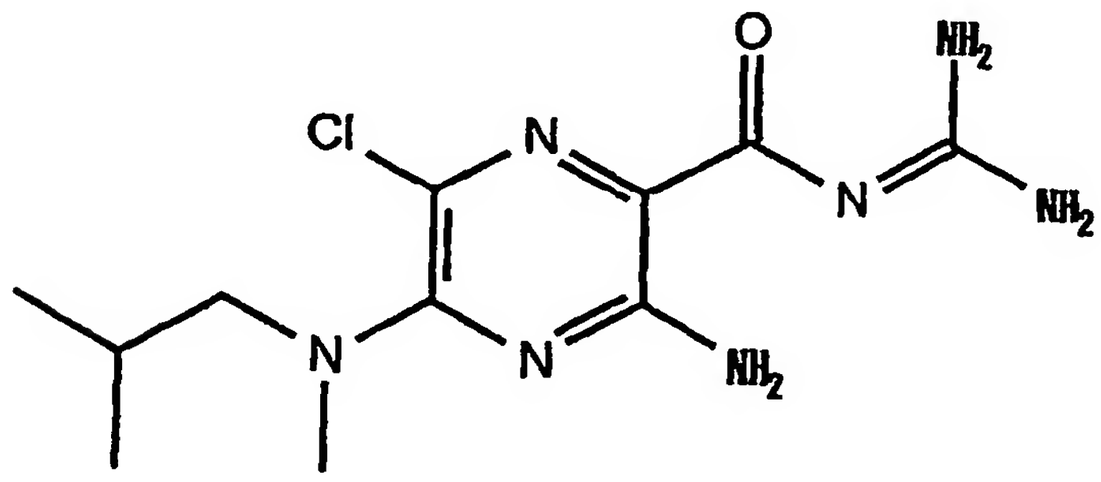
5-(N,N-hexamethylene)amiloride (herein also referred to as HMA)



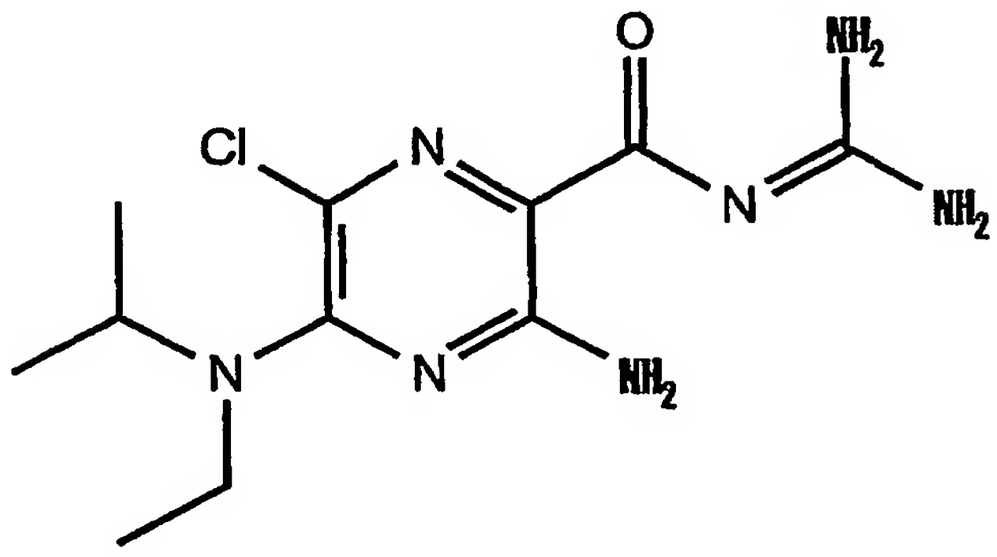
5-(N,N-Dimethyl)amiloride hydrochloride



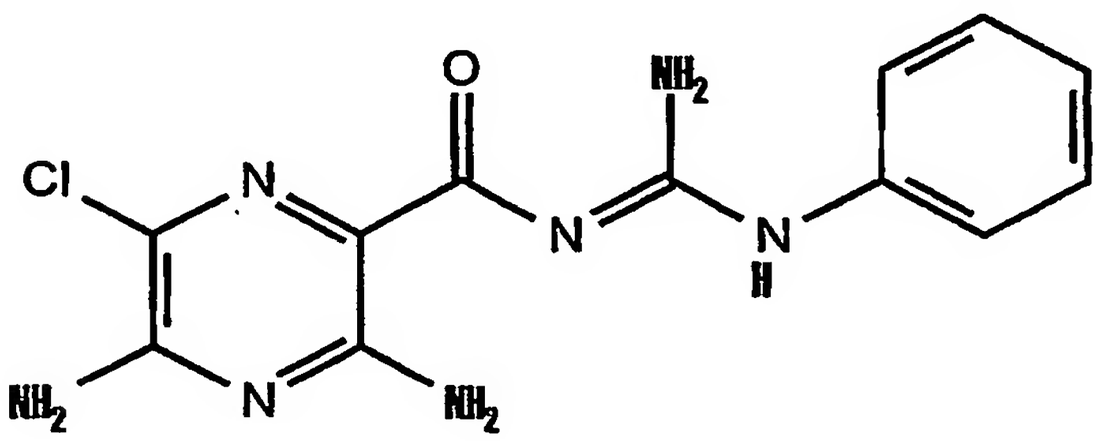
5-(N-methyl-N-isobutyl)amiloride comprising the structure



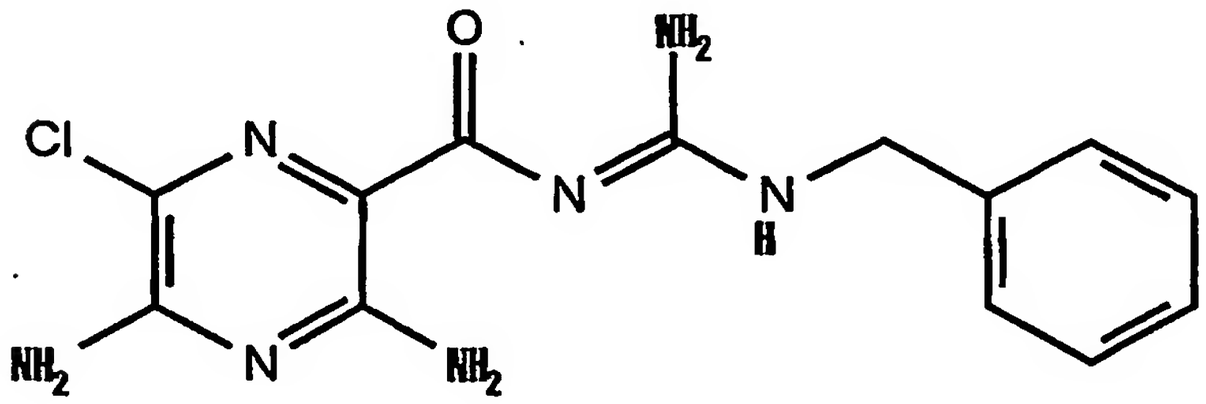
5-(N-ethyl-N-isopropyl)amiloride (herein referred to as EIPA), comprising the structure



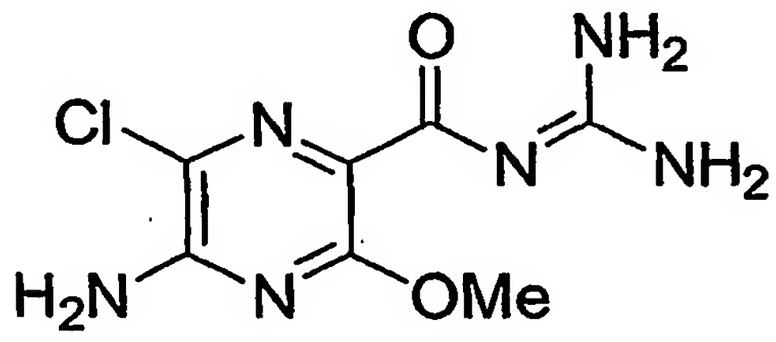
N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine, comprising the structure



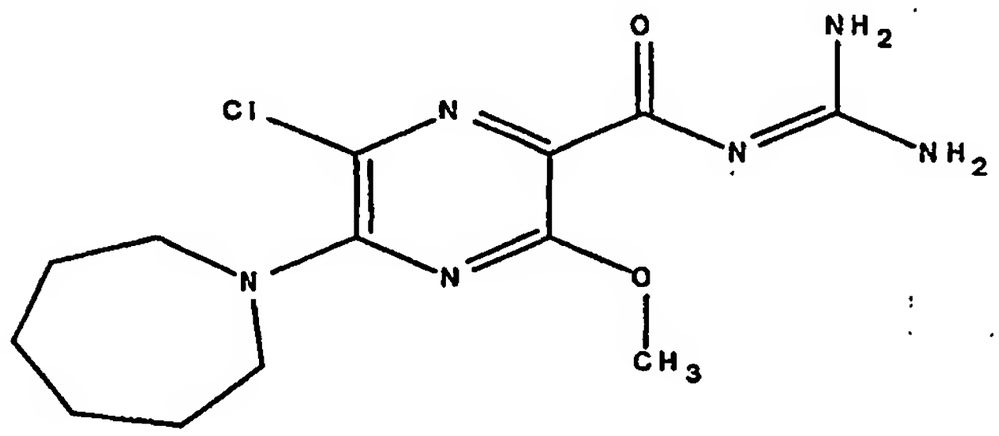
N-Benzyl –N'-(3,5-diamino-6-chloro-pyrzine-2-carbonyl)-guanidine, comprising the structure



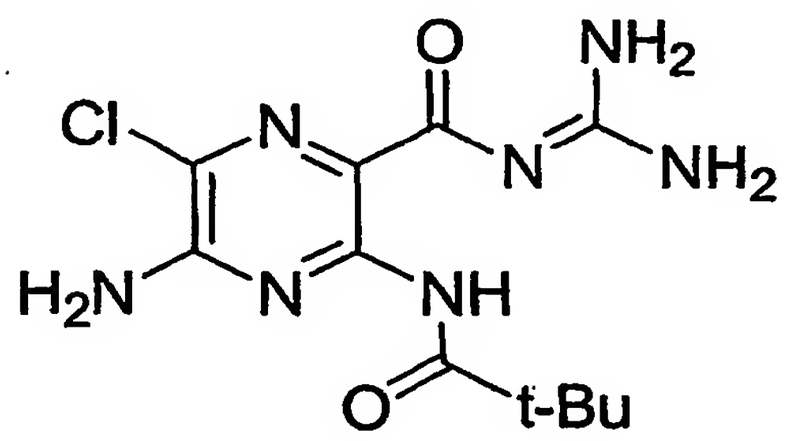
3-methoxy amiloride comprising the structure



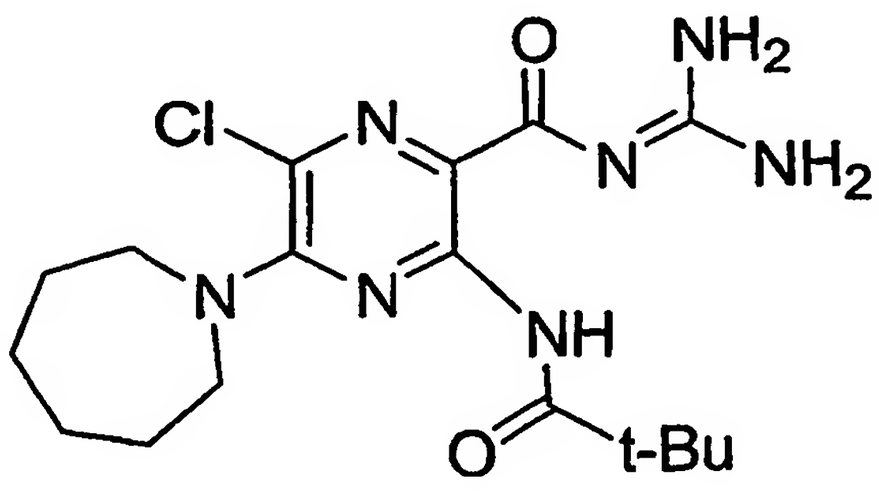
3-methoxy-5-(N,N-Hexamethylene)-amiloride comprising the structure



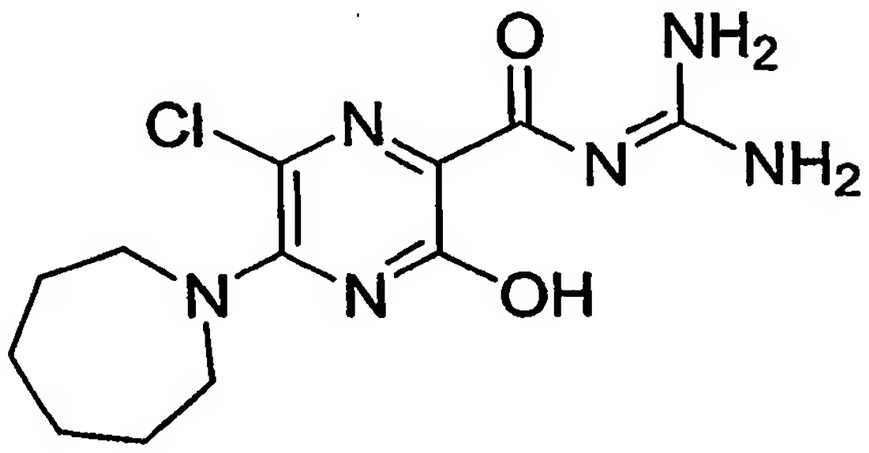
3-(N-2,2 -dimethyl propanal)amiloride comprising the structure



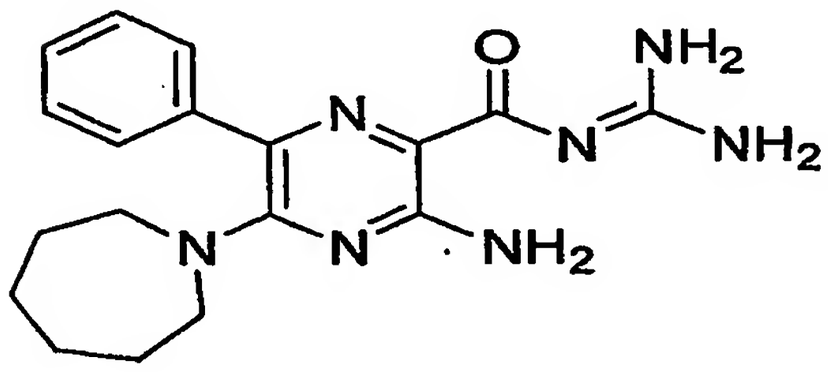
3-(N-2,2 -dimethyl propanal)-5-N-hexamethylene amiloride comprising the structure



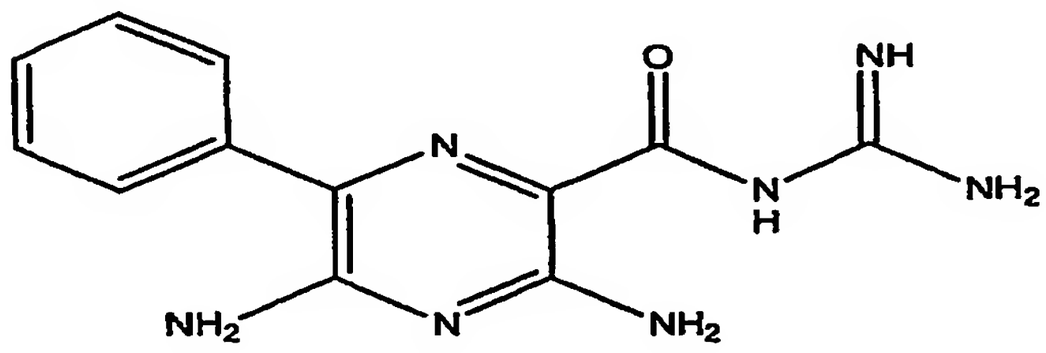
3-hydroxy-5-hexamethyleneimino-amiloride comprising the structure



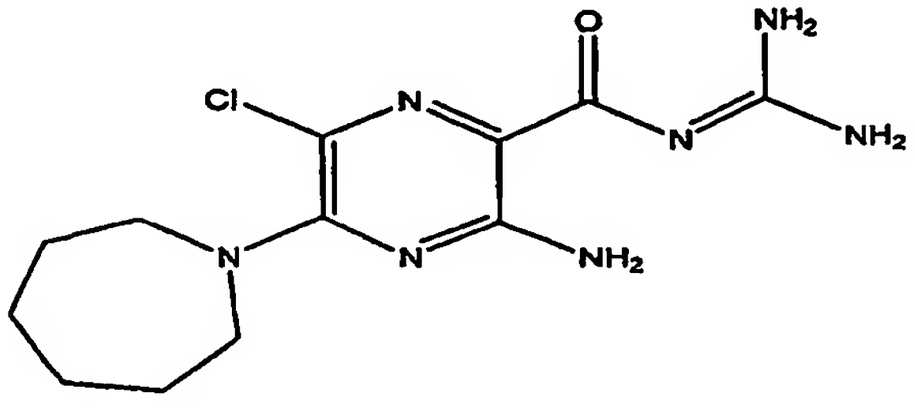
Hexamethyleneimino-6-phenyl-2-pyraxinecarboxamide comprising the structure



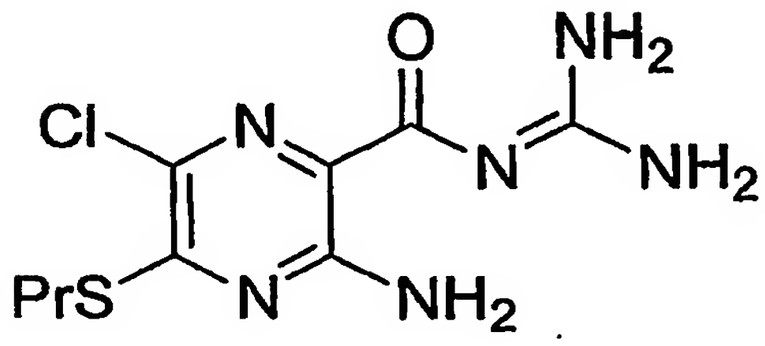
N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide comprising the structure



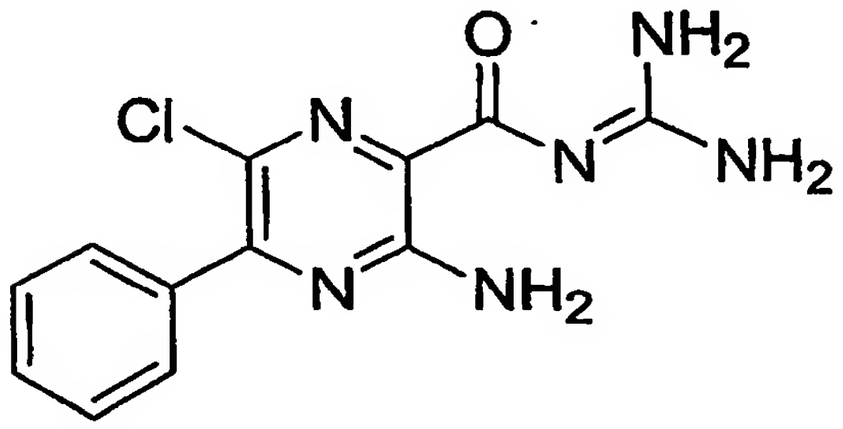
5-(N,N-hexamethylene)amiloride comprising the structure



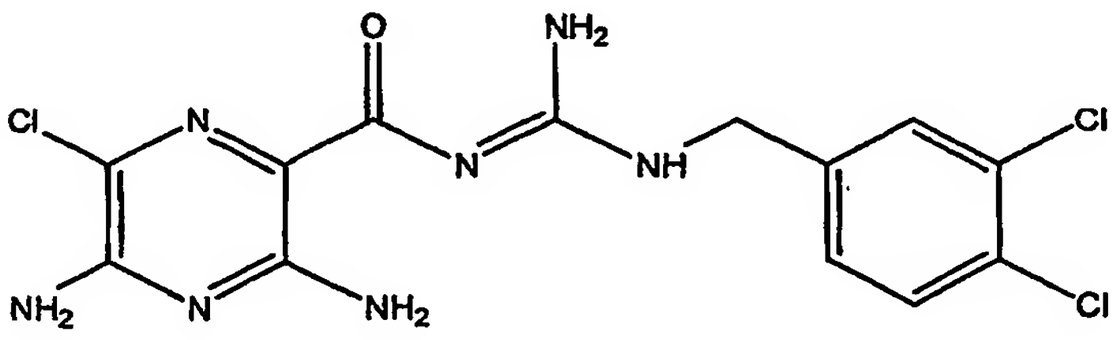
5-propyl-sulfide amiloride comprising the structure



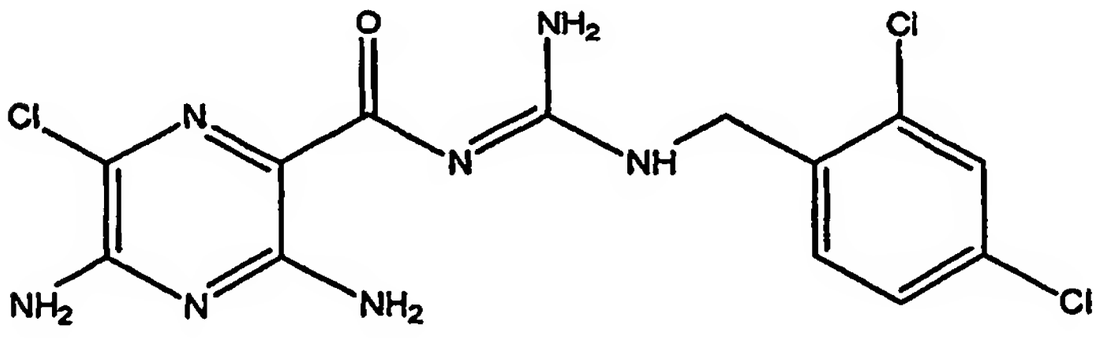
N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide comprising the structure



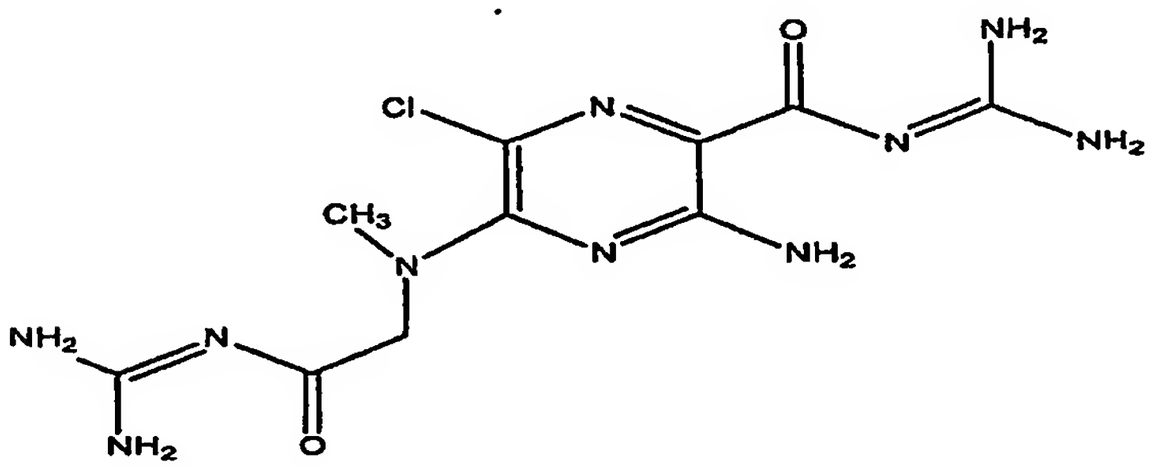
3'4 DichloroBenzamil comprising the structure



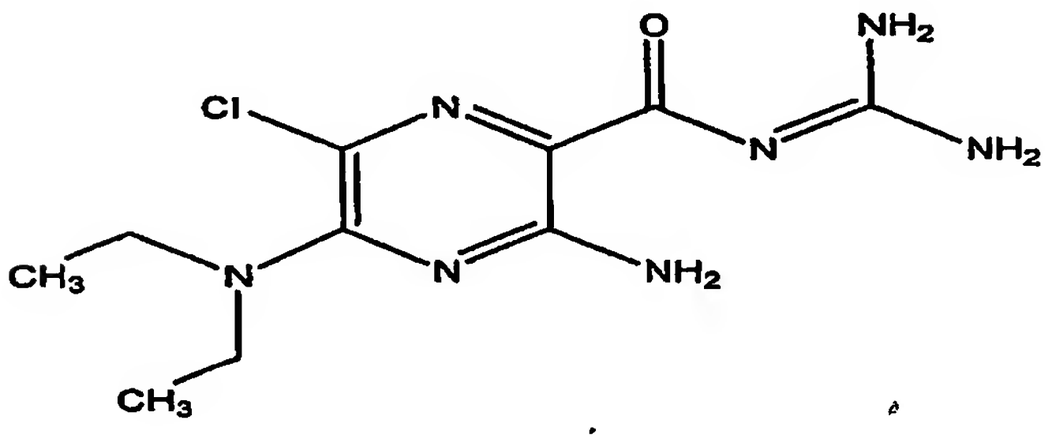
2'4 DichloroBenzamil HCl comprising the structure



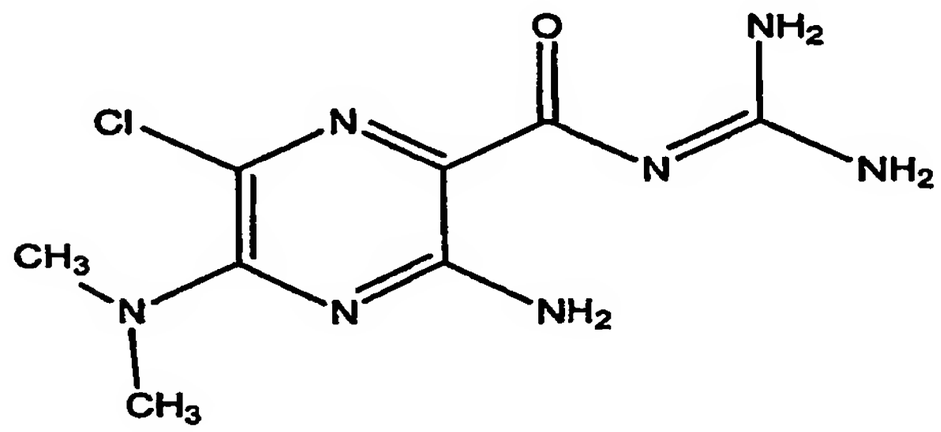
5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride comprising the structure



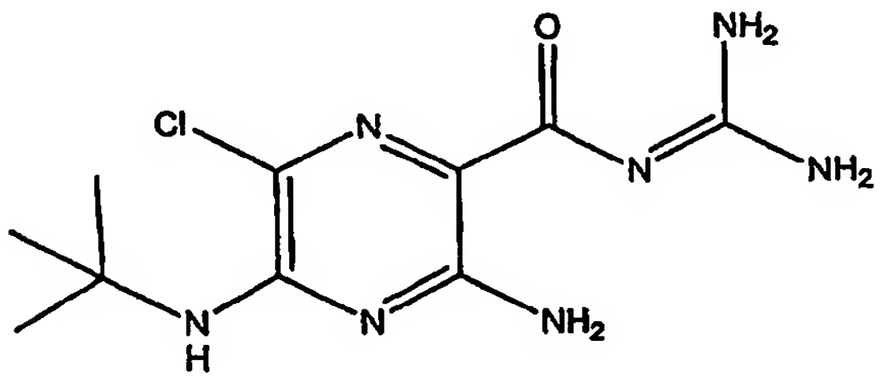
5-(N,N-Diethyl)amiloride hydrochloride comprising the structure



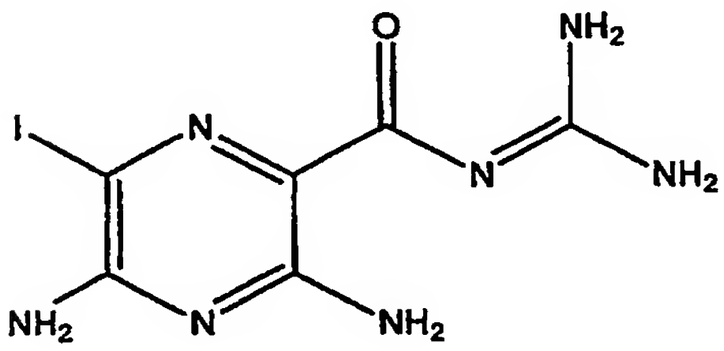
5-(N,N-Dimethyl)amiloride hydrochloride comprising the structure



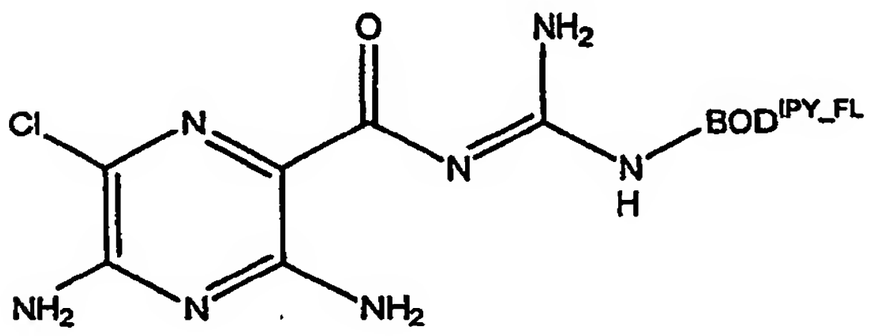
5-tert-butylamino-amiloride comprising the structure



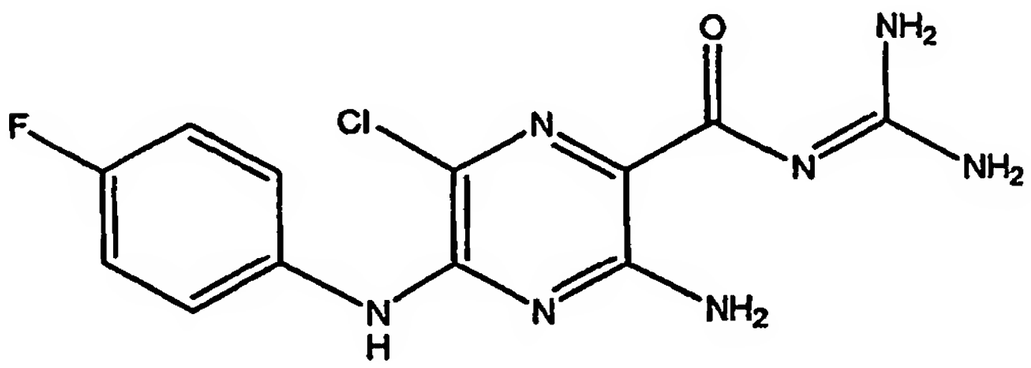
6-Iodoamiloride comprising the structure



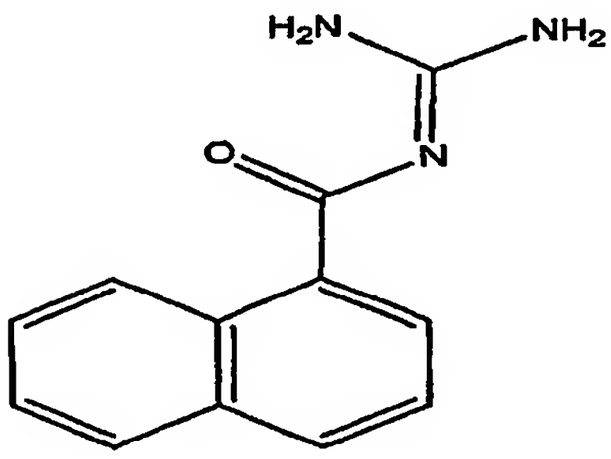
Bodipy-FL Amiloride comprising the structure



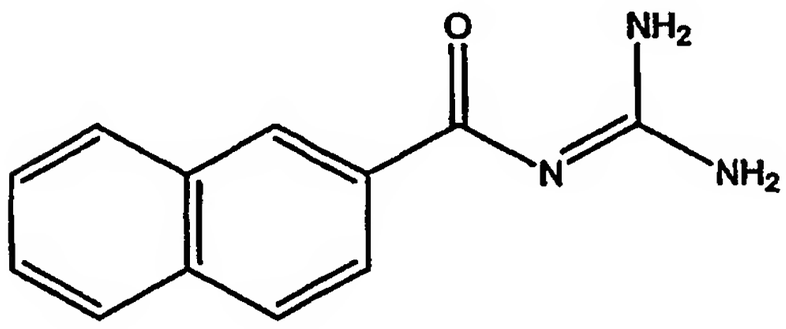
5-(4-fluorophenyl)amiloride comprising the structure



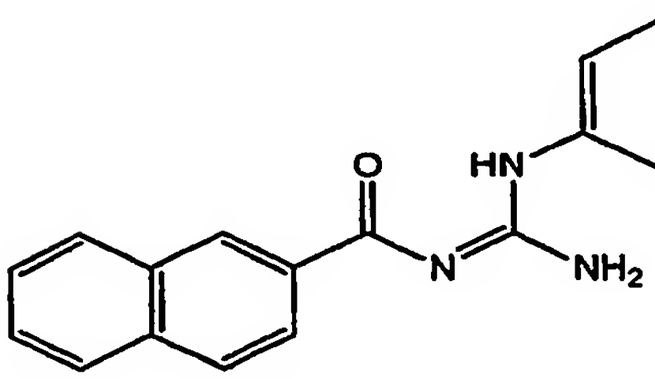
1-napthoylguanidine comprising the structure



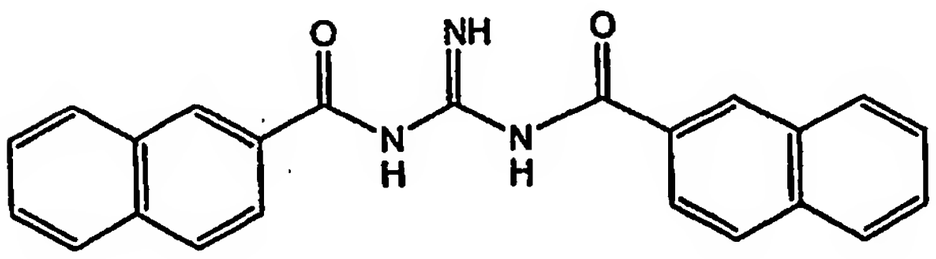
2-napthoylguanidine comprising the structure



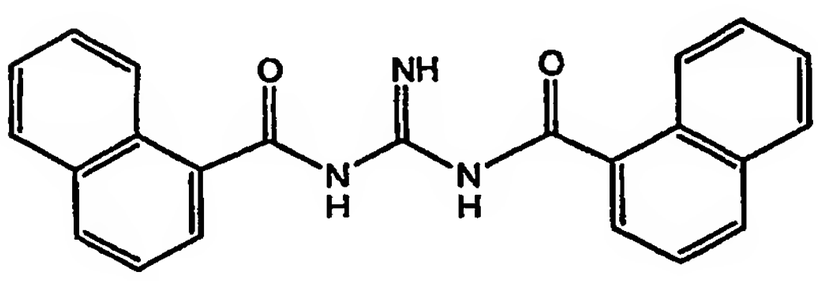
N-(2-napthoyl)-N'-phenylguanidine comprising the structure



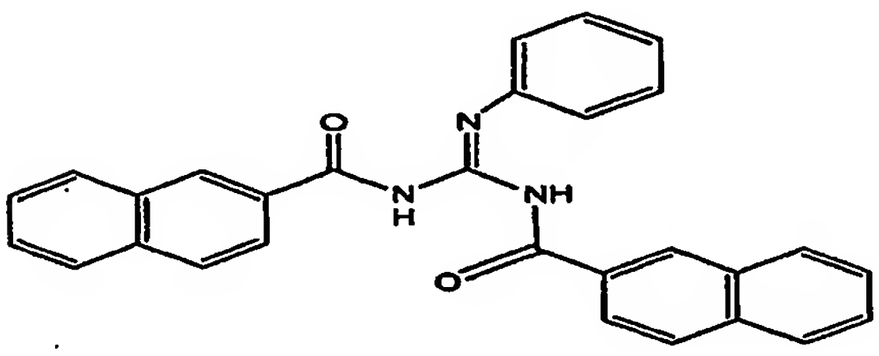
N,N'-bis(2-napthoyl)guanidine comprising the structure



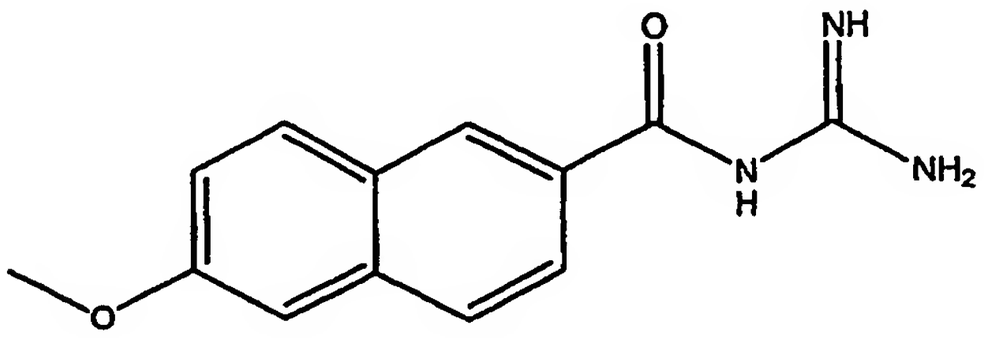
N,N'-bis(1-napthoyl)guanidine comprising the structure



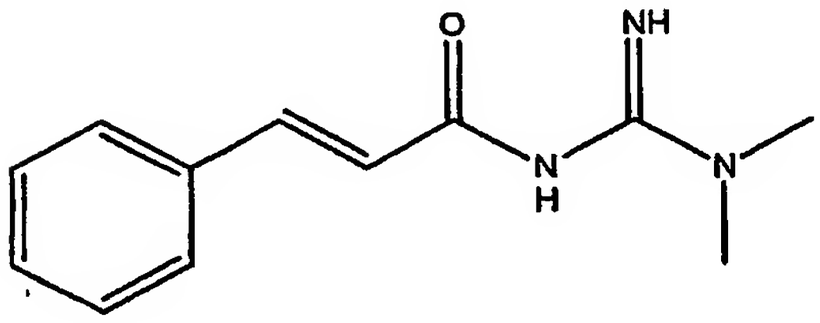
N,N'-bis(2-napthoyl)-N''-phenylguanidine comprising the structure



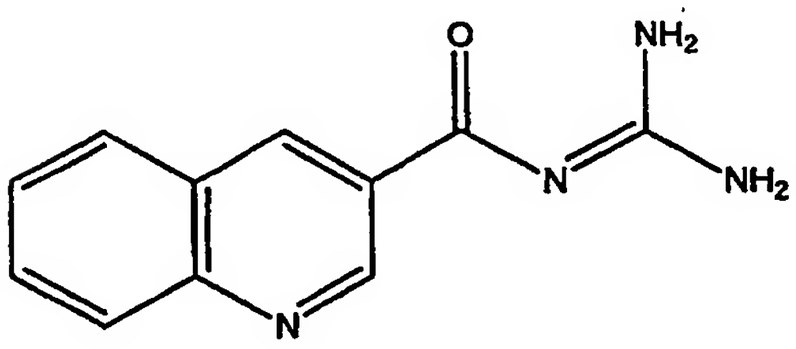
6-methoxy-2-naphthoylguanidine comprising the structure



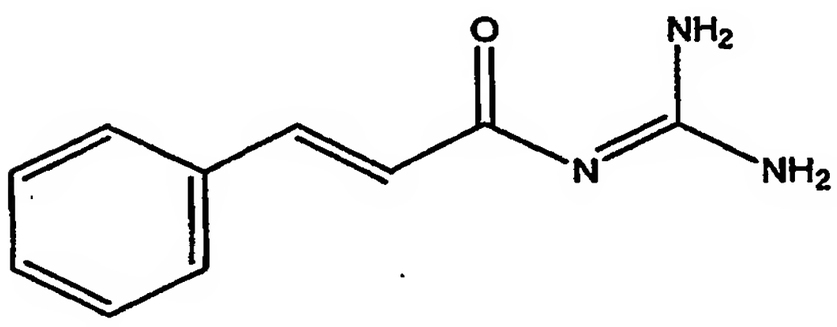
N-Cinnamoyl-N',N'-dimethylguanidine comprising the structure



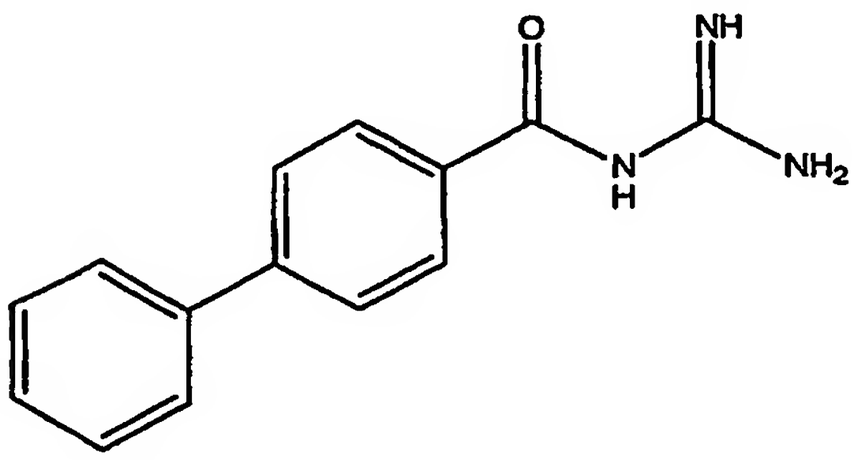
3-quinolinoylguanidine comprising the structure



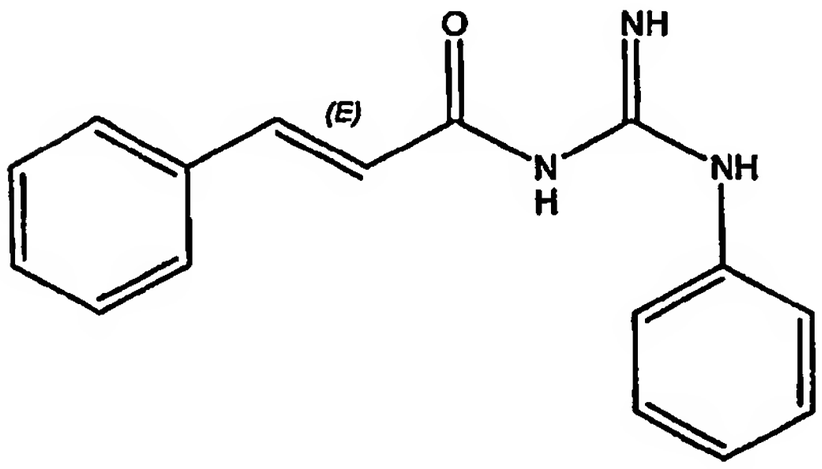
cinnamoylguanidine comprising the structure



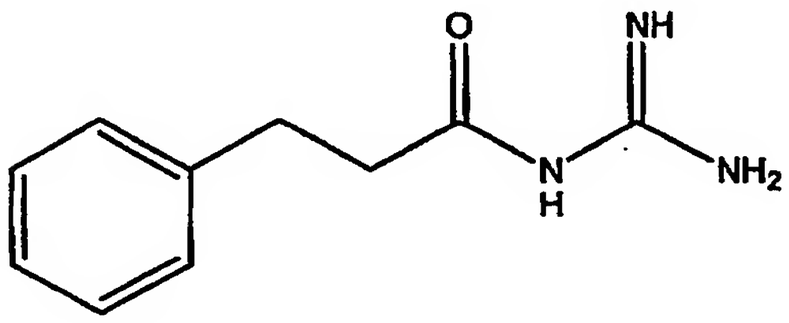
4-phenylbenzoylguanidine comprising the structure



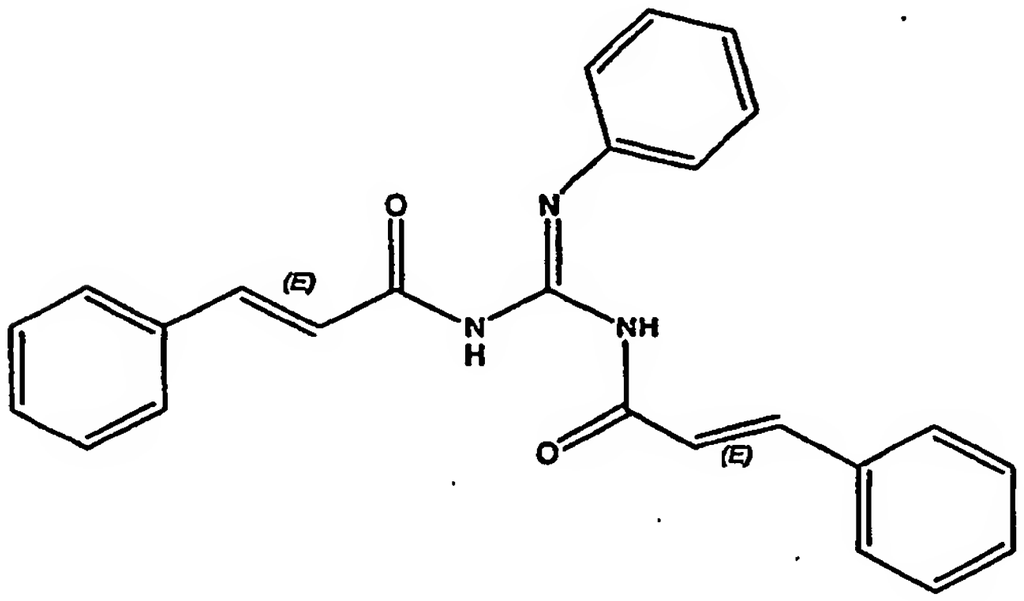
N-(cinnamoyl)-N'phenylguanidine comprising the structure



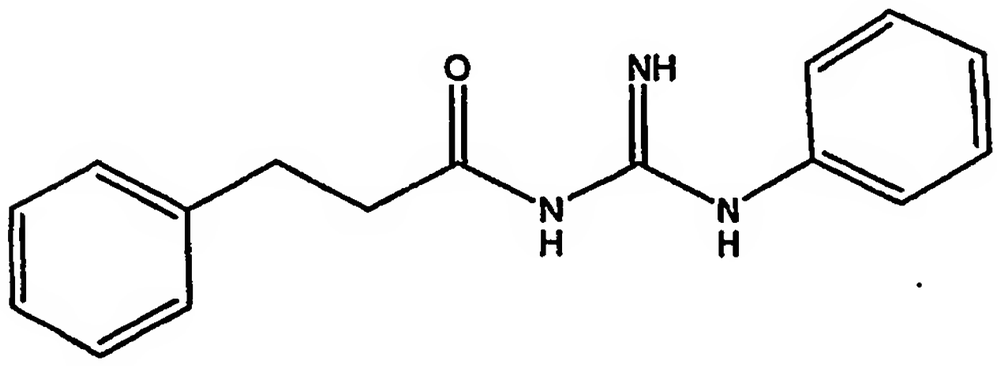
(3-phenylpropanoyl)guanidine comprising the structure



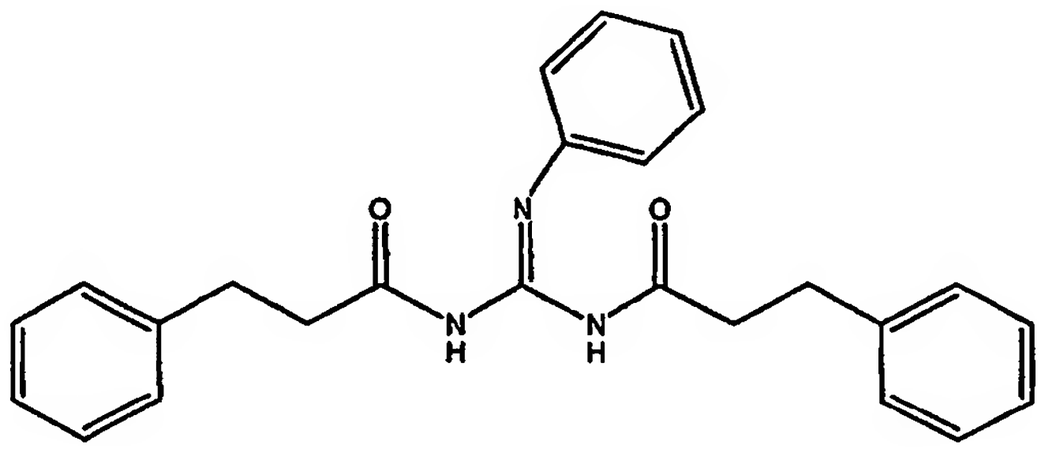
N,N'-bis-(cinnamoyl)-N"-phenylguanidine comprising the structure



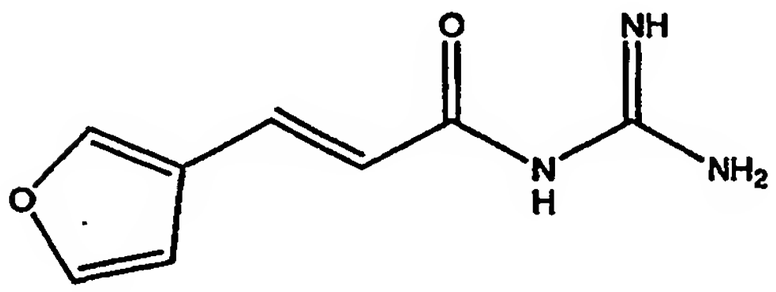
N-(3-phenylpropanoyl)-N'-phenylguanidine comprising the structure



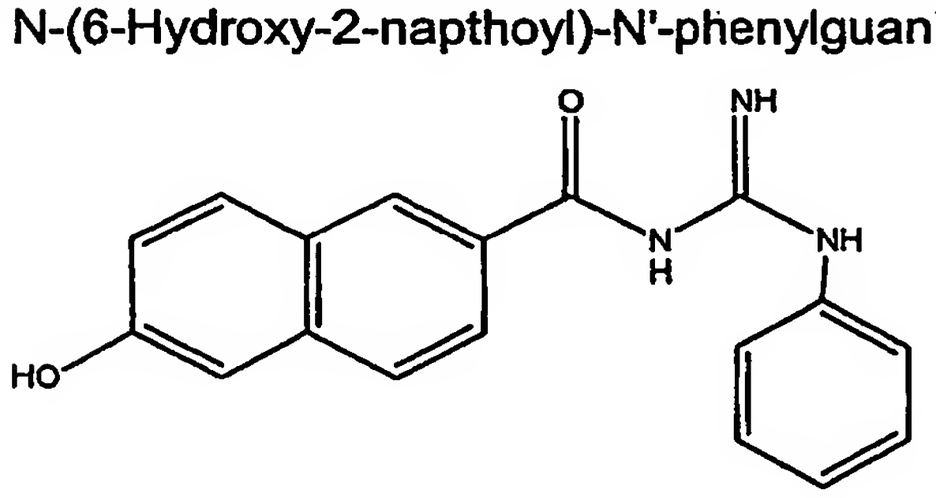
N,N'-bis(3phenylpropanoyl)-N"-phenylguanidine comprising the structure



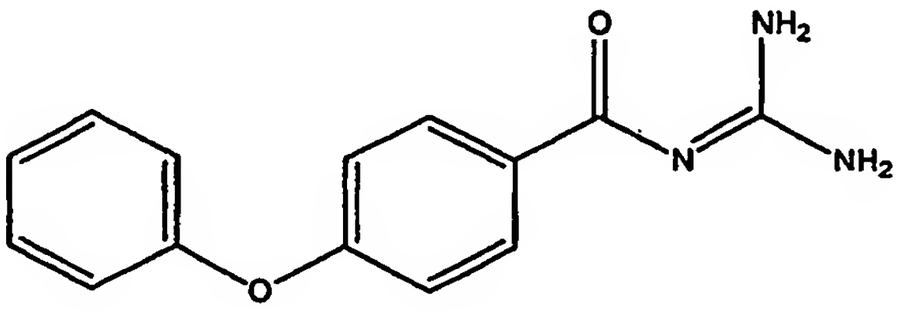
trans-3-furanacryoylguanidine comprising the structure



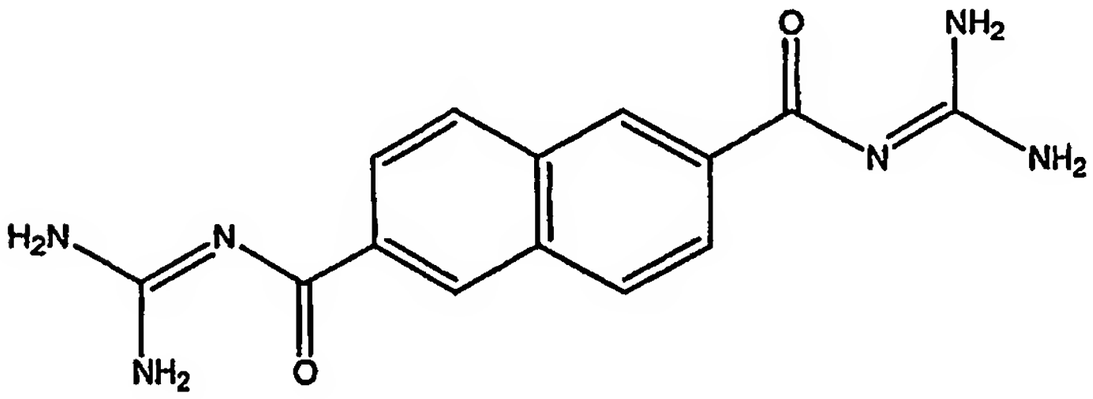
N-(6-Hydroxy-2-napthoyl)-N'-phenylguanidine comprising the structure



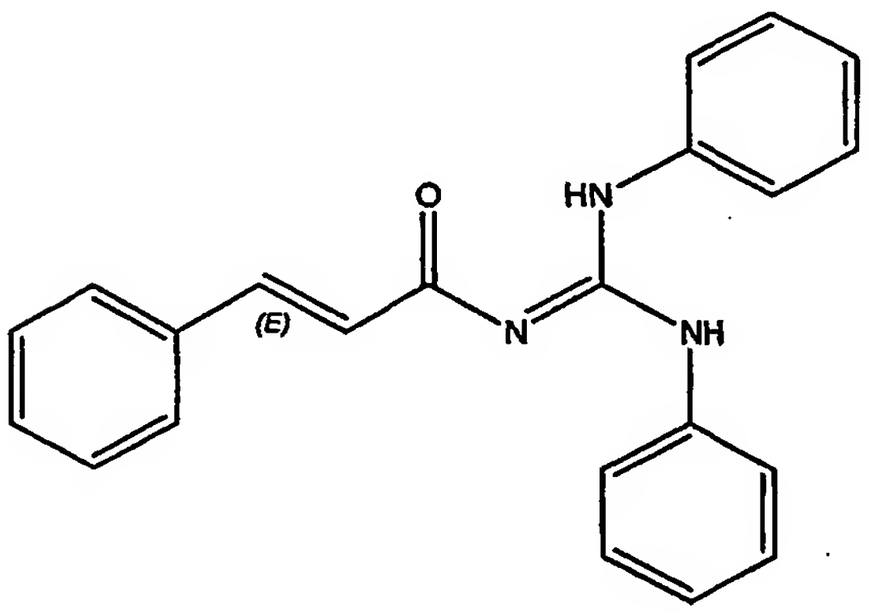
(4-Phenoxybenzoyl)guanidine comprising the structure



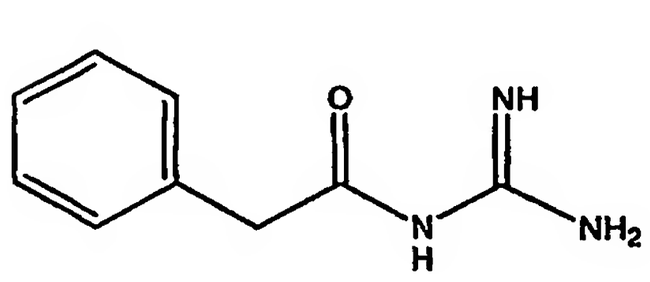
N,N'-Bis(amidino)napthalene-2,6-dicarboxamide comprising the structure



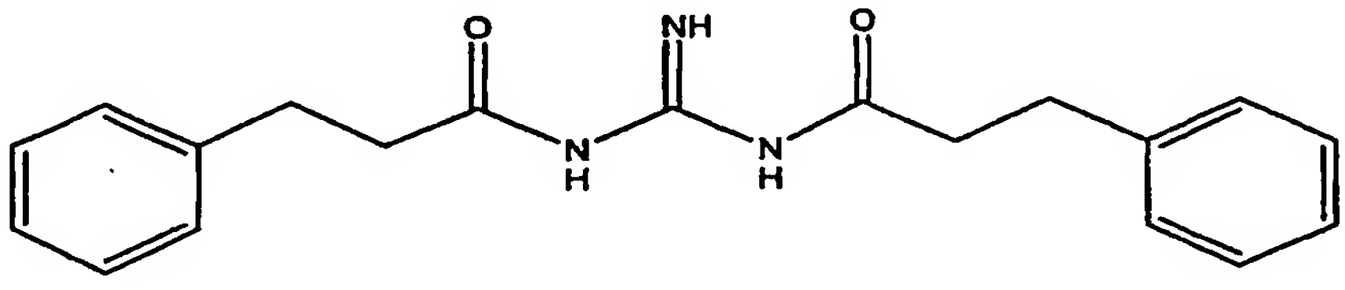
N''-Cinnamoyl-N,N'-diphenylguanidine comprising the structure



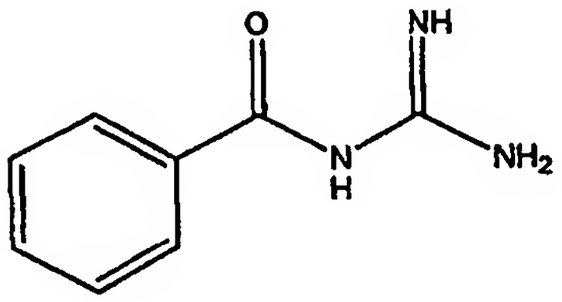
(Phenylacetyl)guanidine comprising the structure



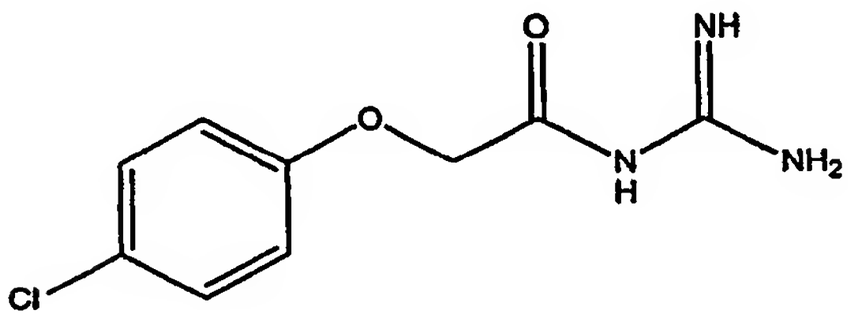
N,N'-Bis(3-phenylpropanoyl)guanidine comprising the structure



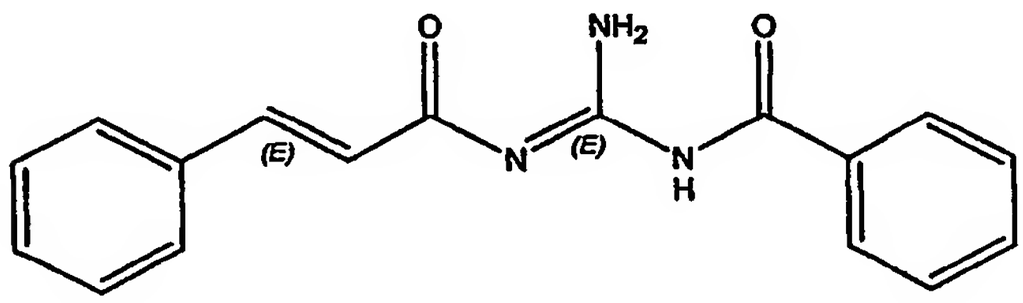
Benzyoylguanidine comprising the structure



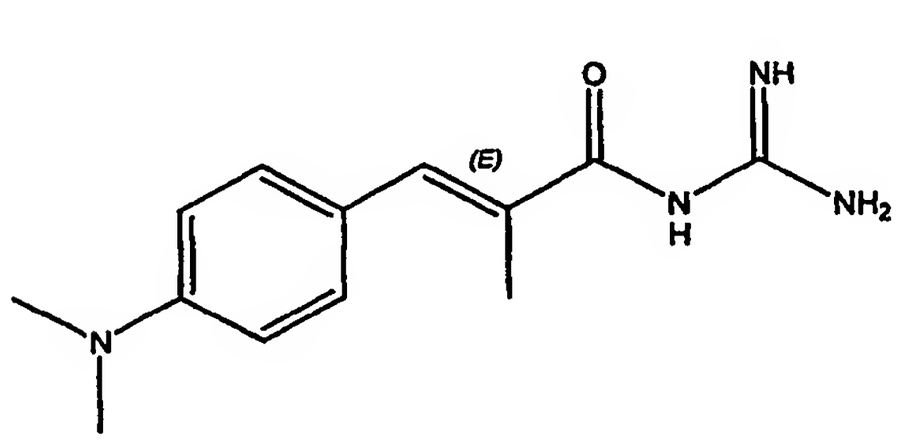
(4-Chlorophenoxy-acetyl]guanidine comprising the structure



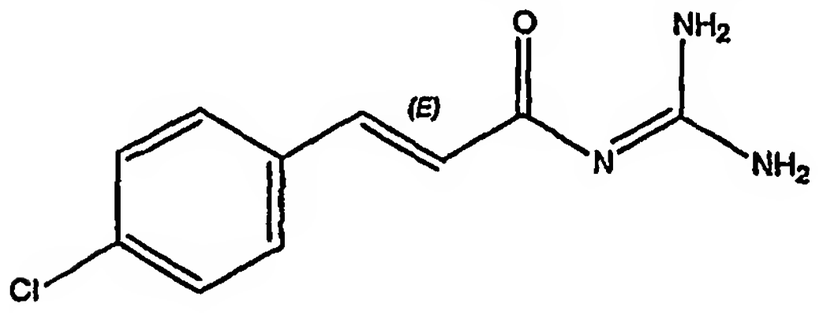
N-Benzoyl-N'-cinnamoylguanidine comprising the structure



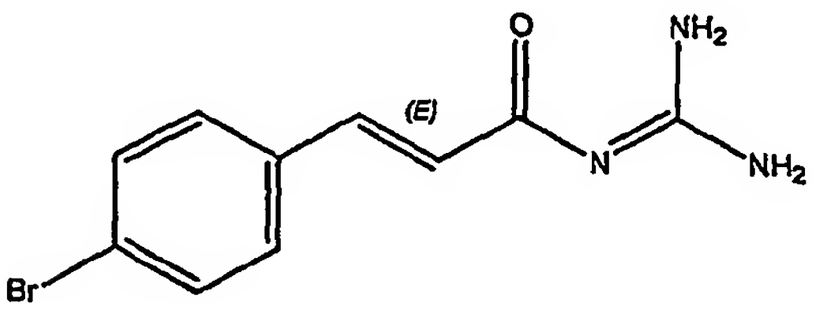
[(E)-3-(4-Dimethylaminophenyl)-2-methylacryloyl]guanidine comprising the structure



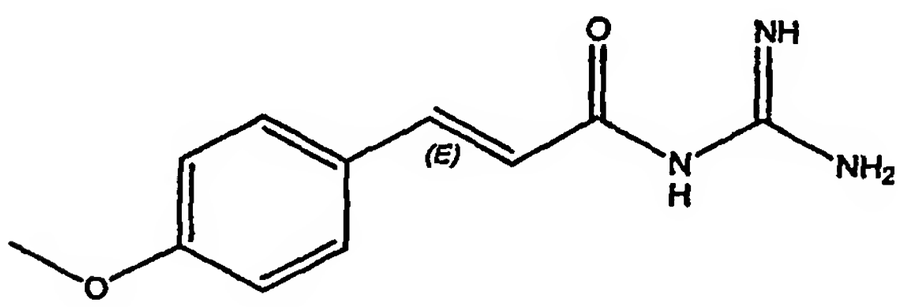
(4-Chlorocinnamoyl)guanidine comprising the structure



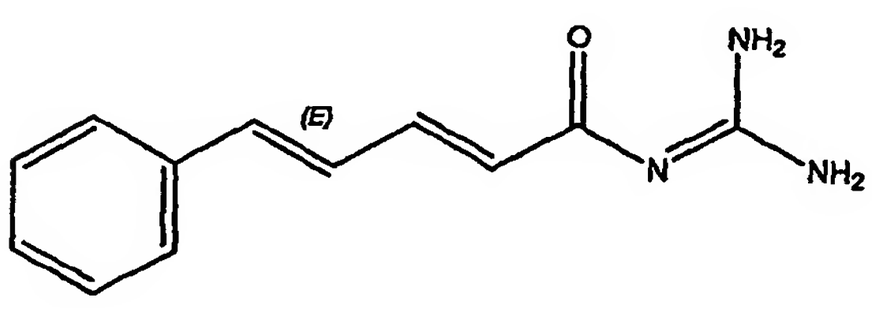
(4-Bromocinnamoyl)guanidine comprising the structure



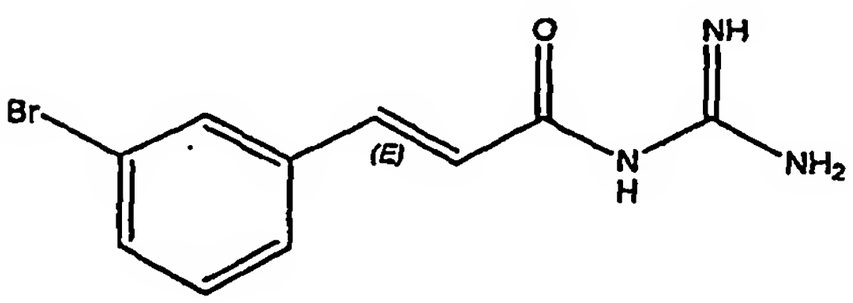
(4-Methoxycinnamoyl)guanidine comprising the structure



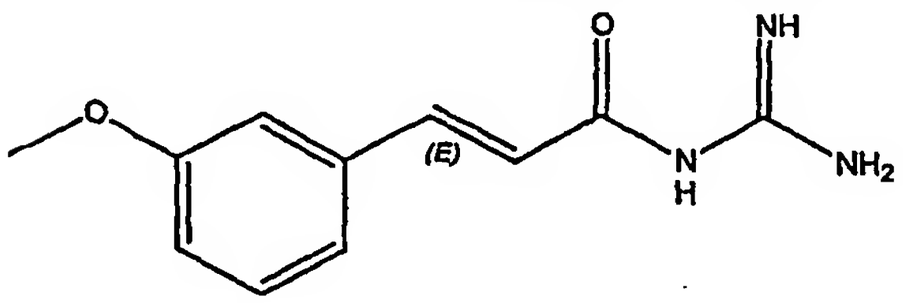
(5-Phenyl-penta-2,4-dienoyl)guanidine comprising the structure



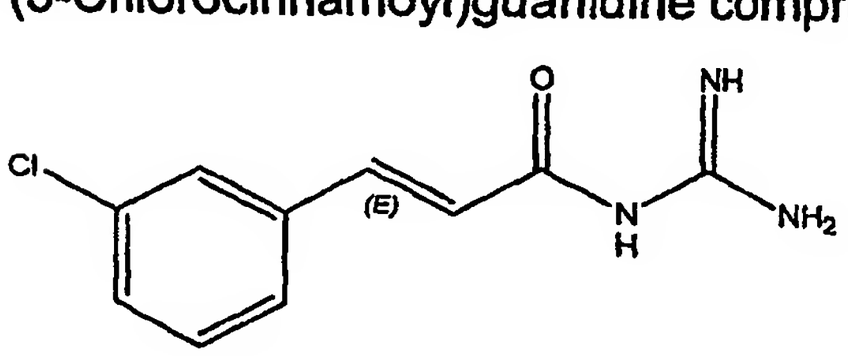
(3-Bromocinnamoyl)guanidine comprising the structure



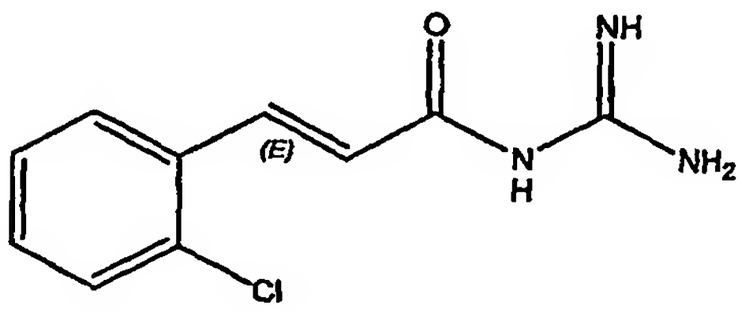
(3-Methoxycinnamoyl)guanidine comprising the structure



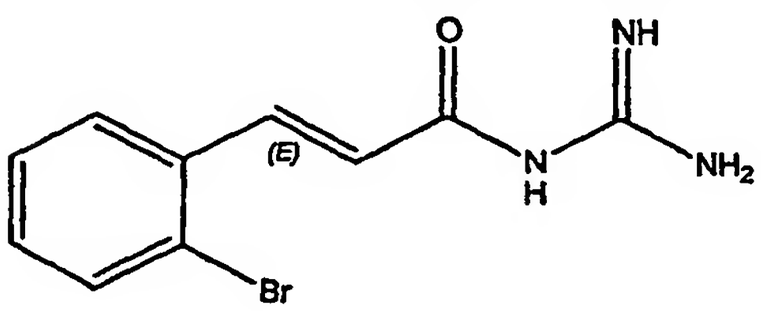
(3-Chlorocinnamoyl)guanidine comprising the structure



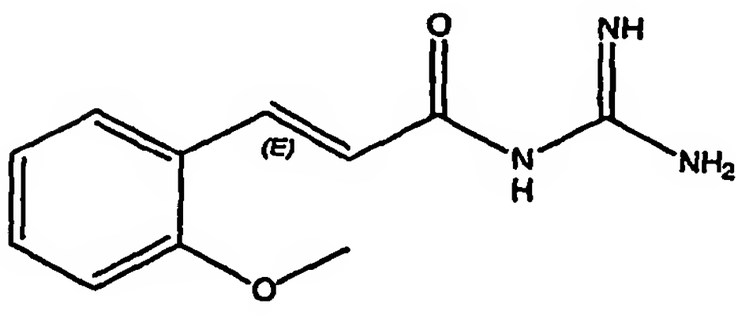
(2-Chlorocinnamoyl)guanidine comprising the structure



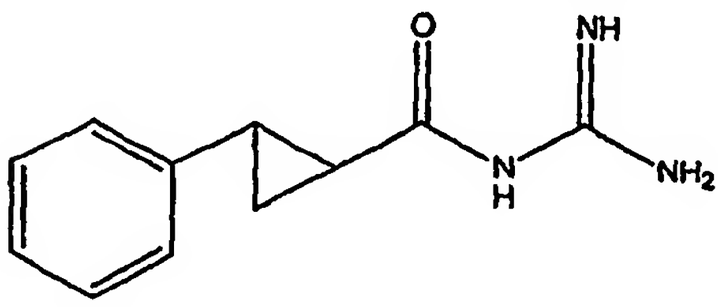
(2-Bromocinnamoyl)guanidine comprising the structure



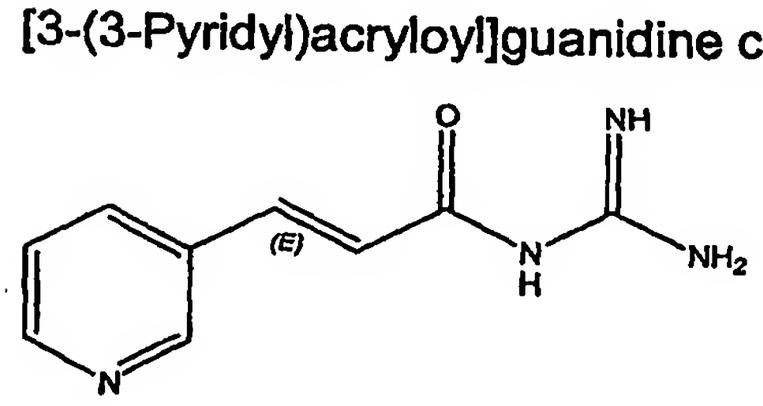
(2-Methoxycinnamoyl)guanidine comprising the structure



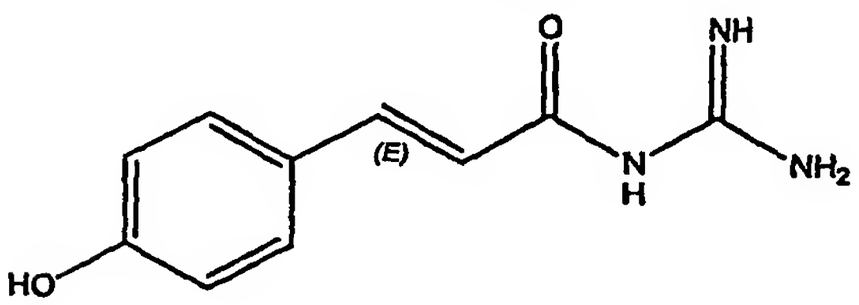
(trans-2-Phenylcyclopropanecarbonyl)guanidine comprising the structure



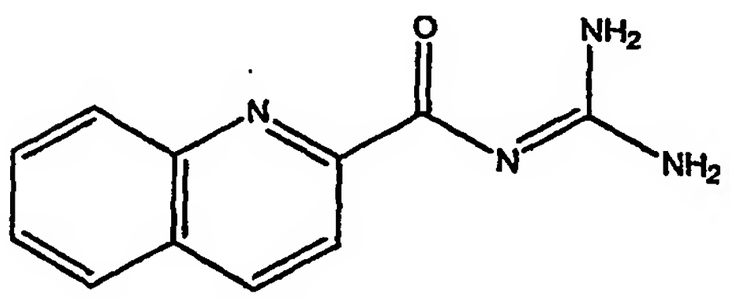
[3-(3-Pyridyl)acryloyl]guanidine comprising the structure



(4-Hydroxycinnamoyl)guanidine comprising the structure

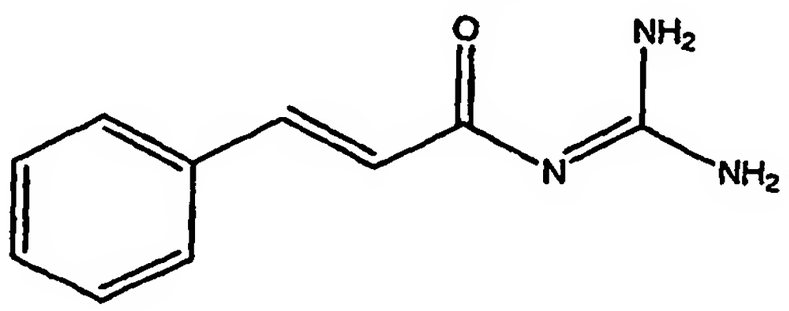


(Quinoline-2-carbonyl)guanidine comprising the structure



One of the preferred compounds of the invention comprises the structure

cinnamoylguanidine (Bit036).



BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Ion channel activity of the C-terminus peptide of Dengue M protein, observed in KCl solutions after incorporation of the peptide into a lipid bilayer. The closed state is shown as a solid line, openings are derivations from the line. Scale bar is 1s and 5pA. The CIS chamber contained 500mM KCl and the TRANS chamber contained 50mM KCl. The potentials were measured in the TRANS chamber relative to the CIS chamber.

Figure 2. Bit009 inhibits ion channel activity of the C-terminus peptide of Dengue M protein in KCl solution. Scale bar is 1s and 4pA. Ion channel activity of the C-terminus peptide of dengue M protein before and after the addition of 100μM Bit009.

DETAILED DESCRIPTION OF THE INVENTION

Flaviviruses are enveloped viruses containing a nucleocapsid that is surrounded by a membrane. The dengue virus, for example, contains a single strand of positive sense RNA that is approximately 19,700 nucleotides. Electron micrographs of the virus show a particle that is about 500A° containing an electron dense core which is surrounded by a lipid bilayer or membrane and a not well defined inner nucleocapsid core (Kuhn et al 2002). There are three structural proteins C (core), M (membrane) and E (envelope).

In the context of the flavivirus genus that includes dengue virus, and without intending to be bound by any particular theory or mechanism of action, the effect of the compounds of the present invention appears to be mediated via the inhibition of, or other negative effect on, the M protein of the dengue virus which was shown in the present studies to behave as an ion channel. As similar M proteins are present across the Flavivirus sub-group of viruses, the compositions and methods of the present invention would have utility in the inhibition and/or treatment of infections by all members of the Flaviviruses, including dengue virus.

The table below provides examples of flaviviruses which could be inhibited or infection treated by the compositions and methods of the present invention.

Table 1 Known Flavivirus Strains

Flavivirus (arboviruses group B)
Dengue virus group
Dengue virus
Dengue virus type 1
Dengue virus type 1 (strain 836-1)
Dengue virus type 1 (strain 924-1)
Dengue virus type 1 (strain AHF 82-80)
Dengue virus type 1 (strain CV1636/77)
Dengue virus type 1 (strain Singapore S275/90)
Dengue virus type 1 (strain TH-SMAN)
Dengue virus type 1 (strain Western Pacific)
Dengue virus type 2
Dengue virus type 2 (isolate Malaysia M1)
Dengue virus type 2 (isolate Malaysia M2)
Dengue virus type 2 (isolate Malaysia M3)
Dengue virus type 2 (NGC-prototype)
Dengue virus type 2 (strain 16681)
Dengue virus type 2 (strain 16681-PDK53)
Dengue virus type 2 (strain D2-04)
Dengue virus type 2 (strain Jamaica)
Dengue virus type 2 (strain PR159/S1)
Dengue virus type 2 (strain PUO-218)
Dengue virus type 2 (strain TH-36)
Dengue virus type 2 (strain Tonga 1974)
Dengue virus type 3
Dengue virus type 4
Japanese encephalitis virus group
Alfuy virus
Japanese encephalitis virus
Japanese encephalitis virus strain JAOARS982
Japanese encephalitis virus strain Nakayama
Japanese encephalitis virus strain SA(V)
Japanese encephalitis virus strain SA-14
Kokobera virus
Koutango virus
Murray Valley encephalitis virus
St. Louis encephalitis virus
St. Louis encephalitis virus (strain MS1-7)
Stratford virus
Usutu virus
West Nile virus
Kunjin virus
Modoc virus group
Cowbone Ridge virus
Jutiapa virus
Modoc virus
Sal Vieja virus

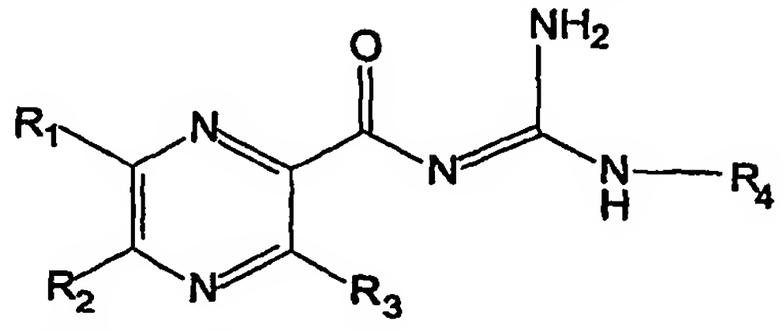
- San Perlita virus
- **mosquito-borne viruses**
  - Ilheus virus
  - Sepik virus
- **Ntaya virus group**
  - Bagaza virus
  - Israel turkey meningoencephalitis virus
  - Ntaya virus
  - Tembusu virus
    - Sitiawan virus
  - Yokose virus
- **Rio Bravo virus group**
  - Apoi virus
  - Bukalasa bat virus
  - Dakar bat virus
  - Entebbe bat virus
  - Rio Bravo virus
  - Saboya virus
- **tick-borne encephalitis virus group**
  - Alkhurma virus
  - Carey Island virus
  - Deer tick virus
  - Karshi virus
  - Kumlinge virus
  - Kyasanur forest disease virus
  - Langat virus
    - Langat virus (strain TP21)
    - Langat virus (strain Yelantsev)
  - Louping ill virus
    - Louping ill virus (strain 31)
    - Louping ill virus (strain K)
    - Louping ill virus (strain Negishi 3248/49/P10)
    - Louping ill virus (strain Norway)
    - Louping ill virus (strain SB 526)
  - Negishi virus
  - Omsk hemorrhagic fever virus
  - Phnom-Penh bat virus
  - Powassan virus
    - Tick-borne powassan virus (strain Ib)
  - Royal Farm virus
  - Skalica virus
  - Tick-borne encephalitis virus
    - Tick-borne encephalitis virus (strain HYPR)
    - Tick-borne encephalitis virus (STRAIN SOFJIN)
  - Turkish sheep encephalitis virus
- **Tyuleniy virus group**
  - Meaban virus
  - Saumarez Reef virus
  - Tyuleniy virus
- **Uganda S virus group**
  - Banzi virus

Bouboui virus
Edge Hill virus
Uganda S virus
Yellow fever virus group
Yellow fever virus
Yellow fever virus (STRAIN 17D)
Yellow fever virus (strain 1899/81)
Yellow fever virus (STRAIN PASTEUR 17D-204)
unclassified Flavivirus
Aroa virus
Batu Cave virus
Bussuquara virus
Cacipacore virus
Flavivirus FSME
Gadgets Gully virus
Greek goat encephalitis virus
Iguape virus
Jugra virus
Kadam virus
Kamiti River virus
Kedougou virus
Montana myotis leukoencephalitis virus
Naranjal virus
Potiskum virus
Rocio virus
Russian Spring-Summer encephalitis virus
Saint Louis encephalitis virus
Sokuluk virus
Spanish sheep encephalitis virus
Spondweni virus
Tamana bat virus
Tick-borne flavivirus
Yaounde virus
Zika virus
Flavivirus sp.
Cell fusing agent virus

The present invention will now be described in more detail with reference to specific but non-limiting examples involving the use of hexamethylene amiloride (Bit009) and the C-terminal peptide of the dengue M protein. It will be clear from the description herein that other flaviviruses and other compounds may be used effectively in the context of the present invention.

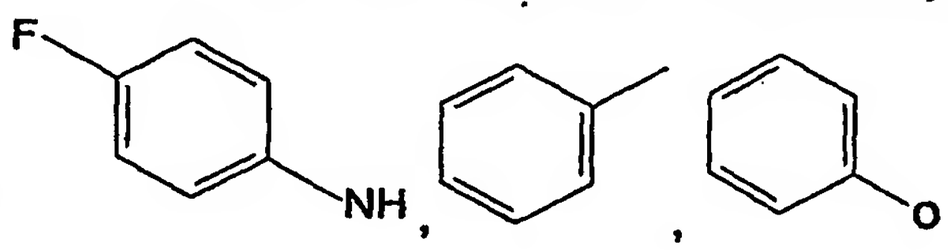
Examples of suitable compounds that can be used in the compositions and methods of the present invention are listed below.

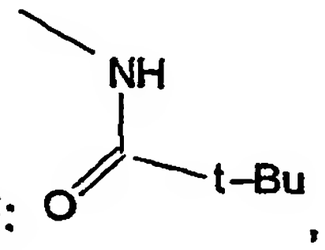
Amiloride analogues or derivatives comprising the structure:

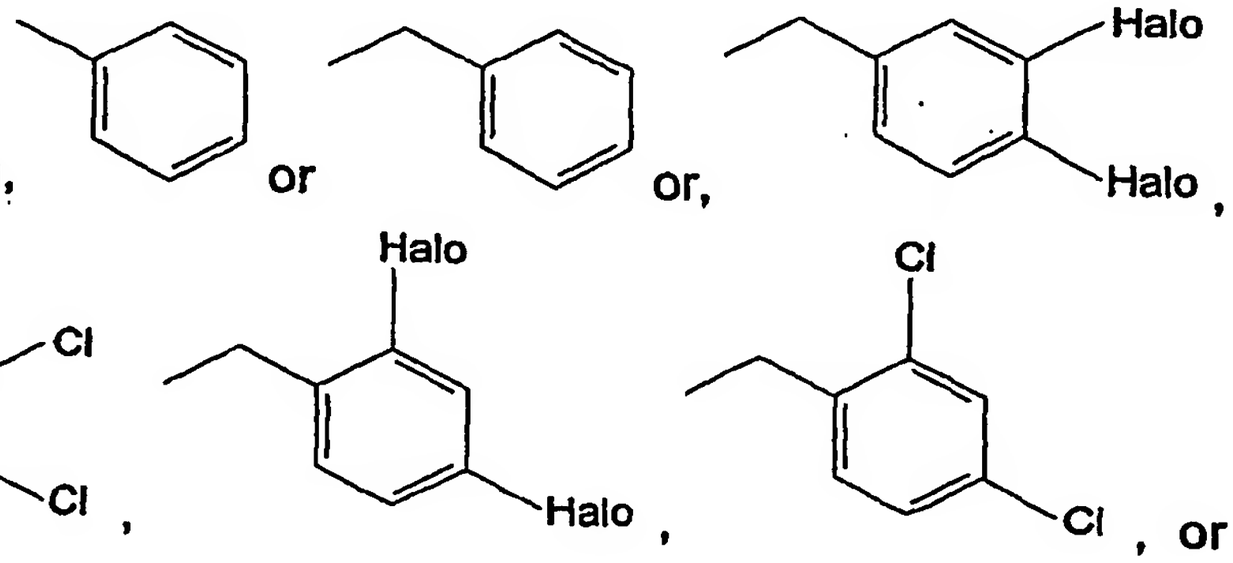


wherein the substituents at the R positions may or may not be the same, and

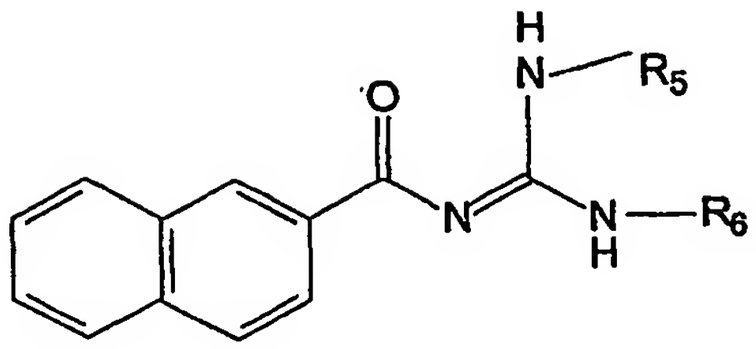
R_1 =hydrogen, halo, aryl, substituted aryl, phenyl, or substituted phenyl;

R_2 = hydrogen, amine, aryl, substituted aryl, halo, phenyl, substituted phenyl, hexamethylene, PrS, N-methyl-N-isobutyl, N-ethyl –N-isopropyl, benzyl ; N-methyl-N-guanidinocarbonyl-methyl, N,N-dimethyl, N,N-diethyl, tert-butylamino, halo-aniline, fluoro-aniline 

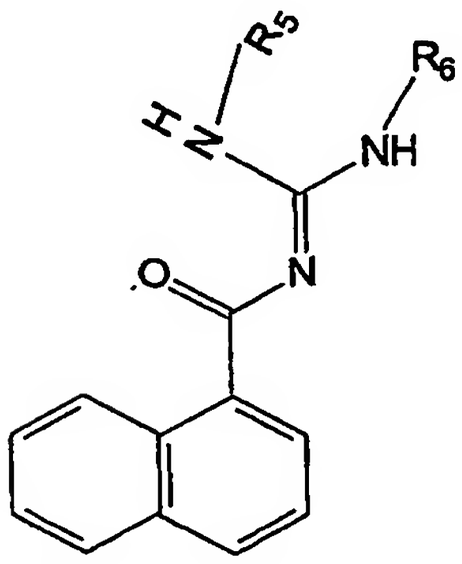
R_3 = hydrogen, hydroxy, halo, alkyloxy, methoxy, N,3-dimethylbutanamyl: 

R_4 = hydrogen,  BOD^{IPY_FL};

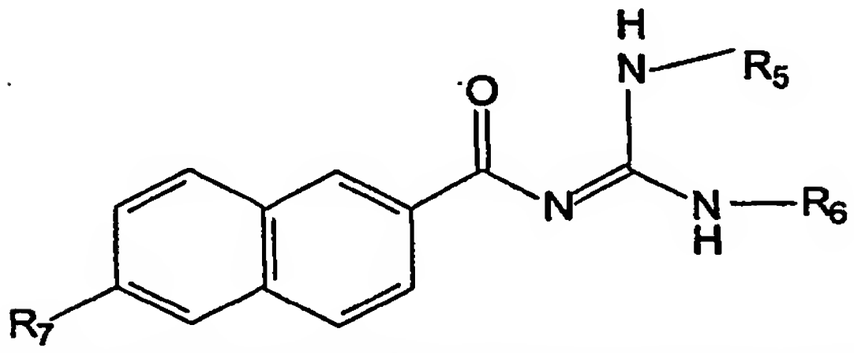
The following antiviral compounds comprising a guanidyl moiety are also encompassed within the scope of the present invention:



or



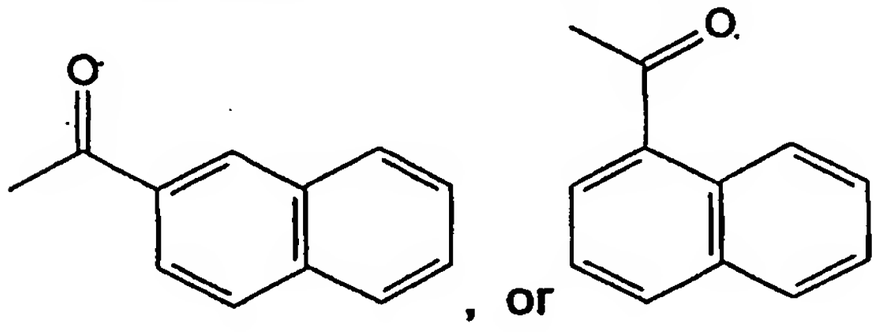
or



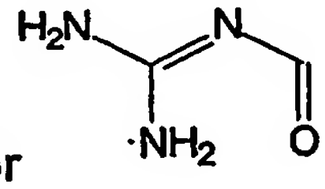
wherein the substitutents at the R positions may or may not be the same, and

R_5= Hydrogen, aryl, substituted aryl, phenyl, or substituted phenyl;

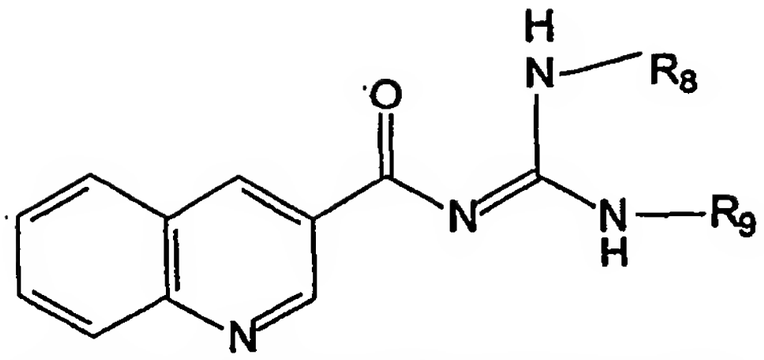
R_6 = Hydrogen, aryl, substituted aryl, phenyl, substituted phenyl, napthoyl,



, or

R_7 = hydrogen, halo, hydroxy , methoxy , alkyloxy , or  ,

or, the structure

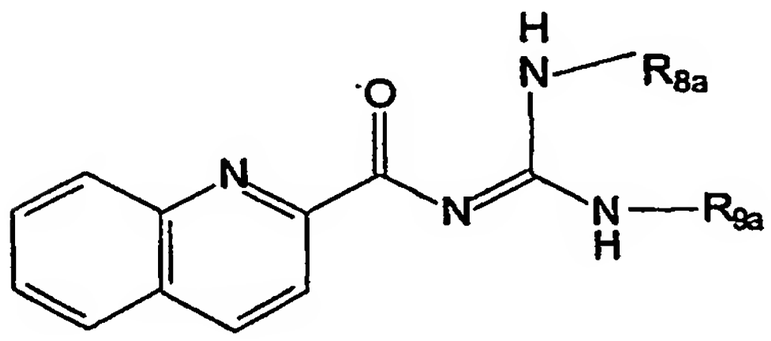


wherein the substitutents at the R positions may or may not be the same, and

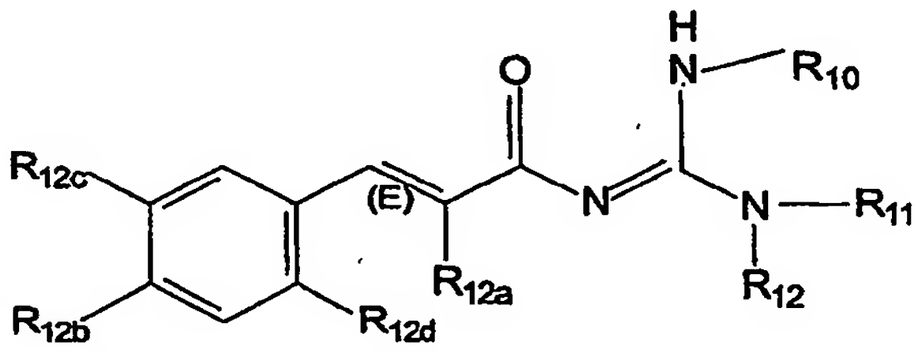
R_8 = aliphatic or aromatic substituents;

R_9 = aliphatic or aromatic substituents;

or the structure

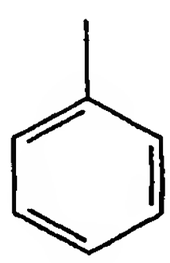
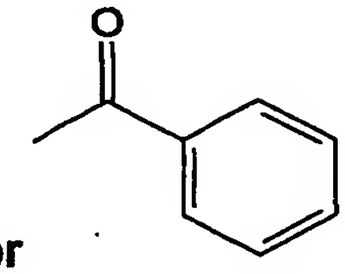


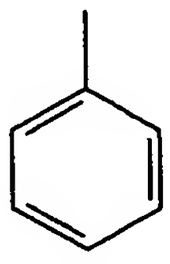
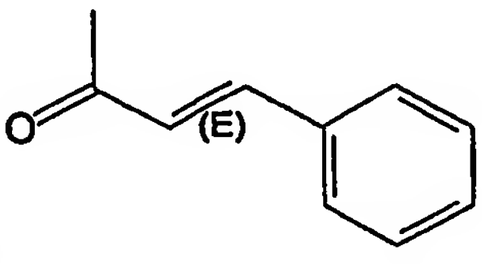
or, the structure



wherein the substitutents at the R positions may or may not be the same, and

R_{10} = hydrogen, aryl, phenyl, or cinnamoyl;

R_{11} = hydrogen , alkyl, aryl, phenyl, cinnamoyl;  , or 

R_{12} = hydrogen, alkyl, aryl, phenyl, cinnamoyl,  , or 

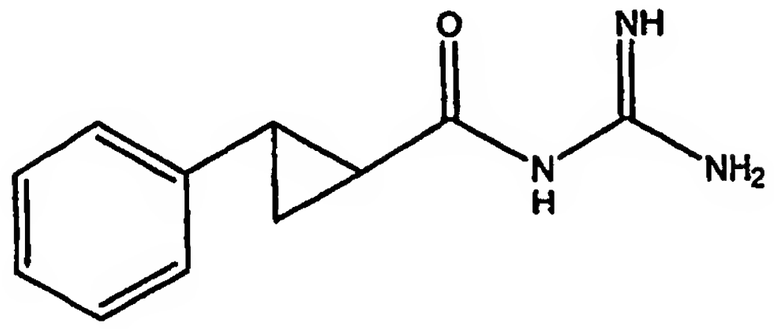
R_{12a} = hydrogen or alkyl,

R_{12b} = hydrogen, halo, hydroxy, alkoxy, or dialkyl amino,

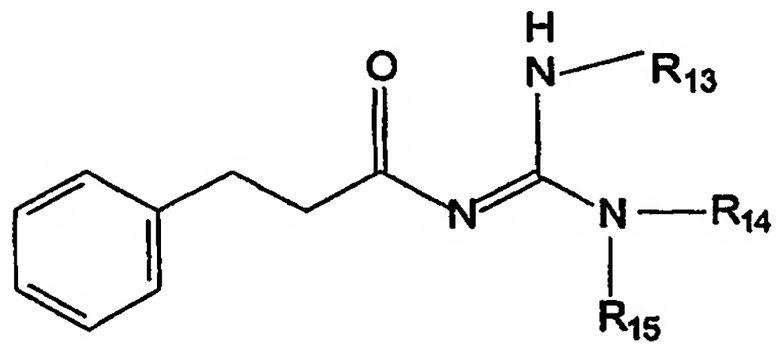
R_{12c} = hydrogen, halo or alkoxy,

R_{12d} = hydrogen, halo or alkoxy,

or the structure,

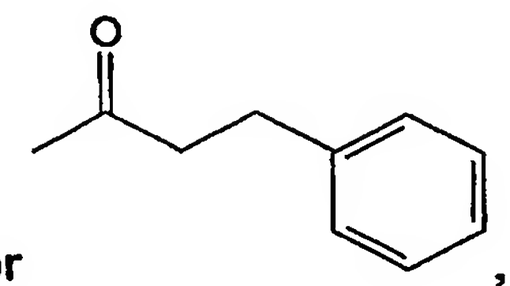


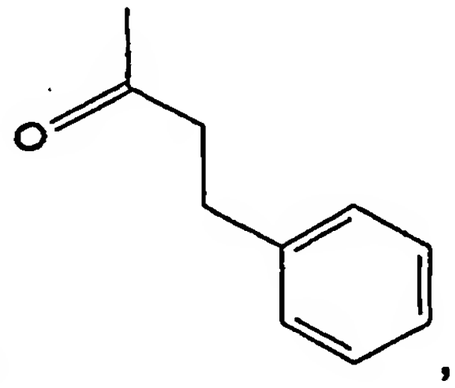
or, the structure



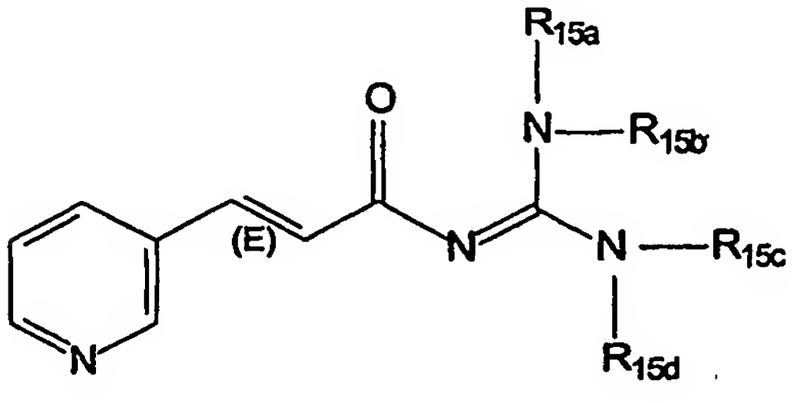
wherein the substitutents at the R positions may or may not be the same, and

R_{13} = H; alkyl, or phenyl

R_{14} = H, alkyl, phenyl, substituted phenyl, or  ,

R_{15} = H, or  ,

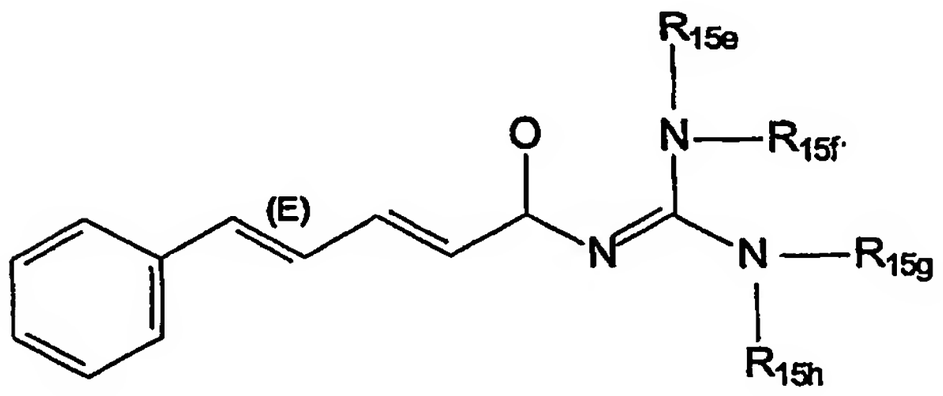
or the structure



wherein the substitutents at the R positions may or may not be the same, and

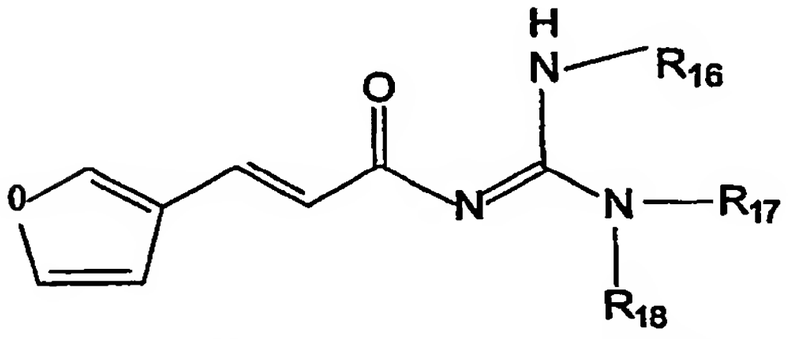
R_{15a}, R_{15b}, R_{15c} and R_{15d} = hydrogen, alkyl, or phenyl

or the structure



R_{15e}, R_{15f}, R_{15g} and R_{15h} = hydrogen; alkyl, or phenyl,

or the structure



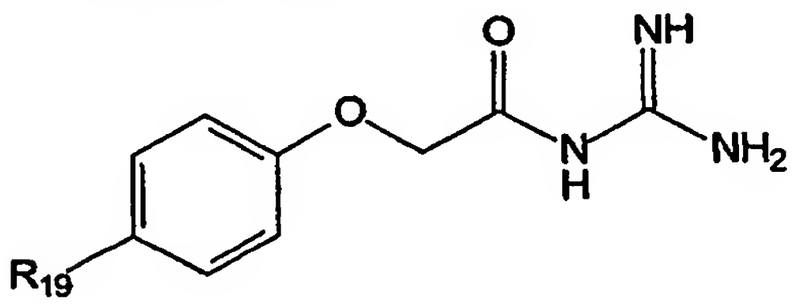
wherein the substitutents at the R positions may or may not be the same, and

R_{16} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

R_{17} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

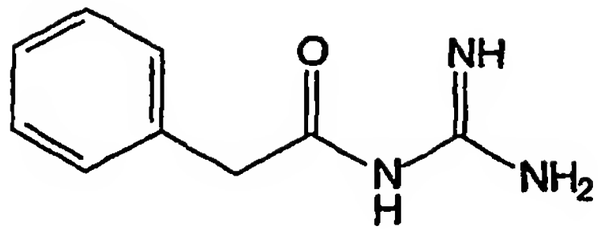
R_{18} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

or the structure



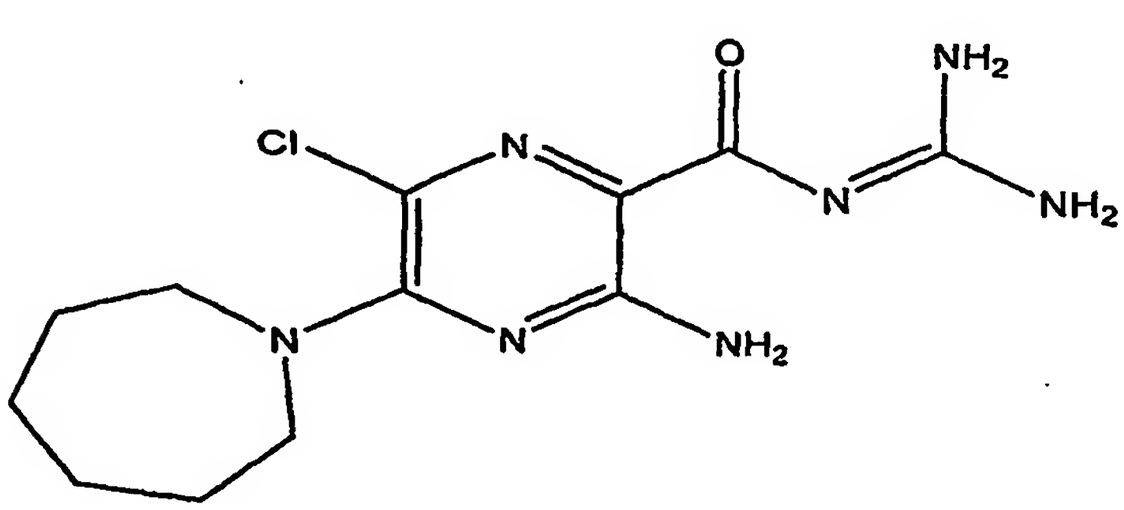
wherein R_{19} = hydrogen, halo; alkyl, hydroxy, alkoxy,

or the structure

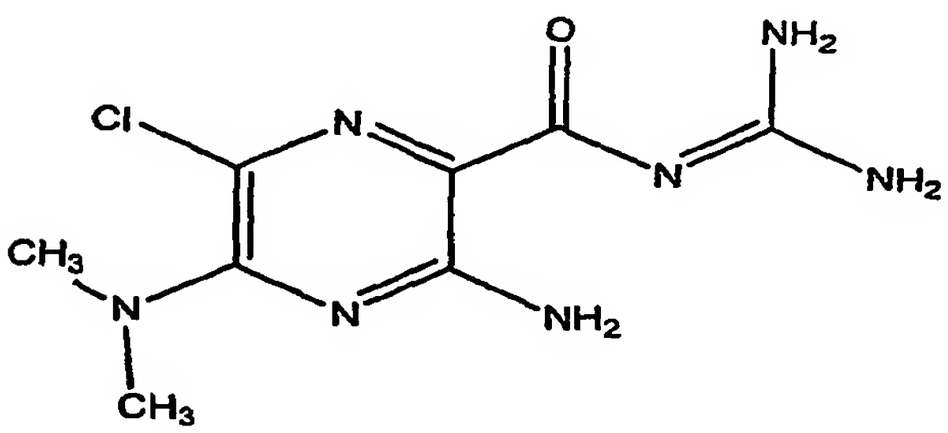


The compounds of the invention include the following:

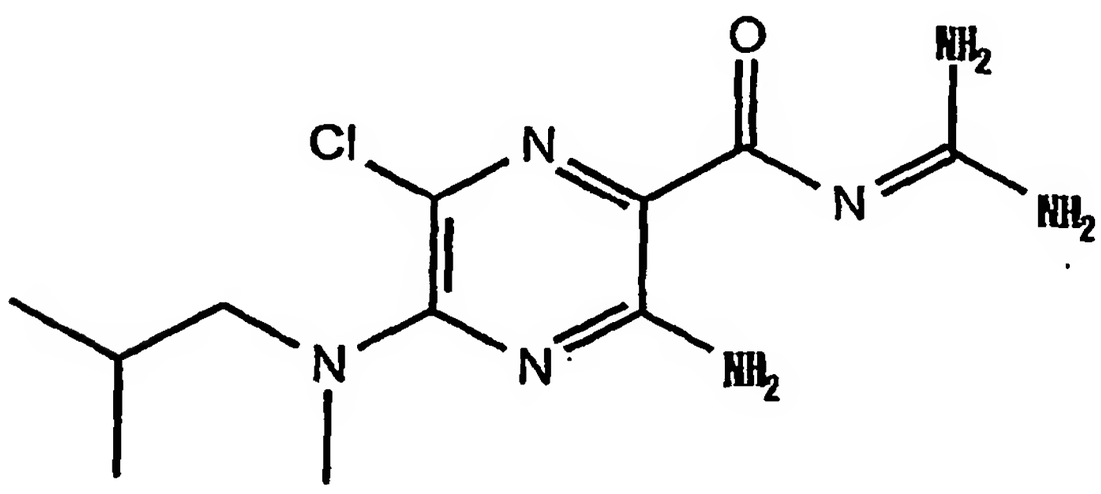
5-(N,N-hexamethylene)amiloride (herein also referred to as HMA)



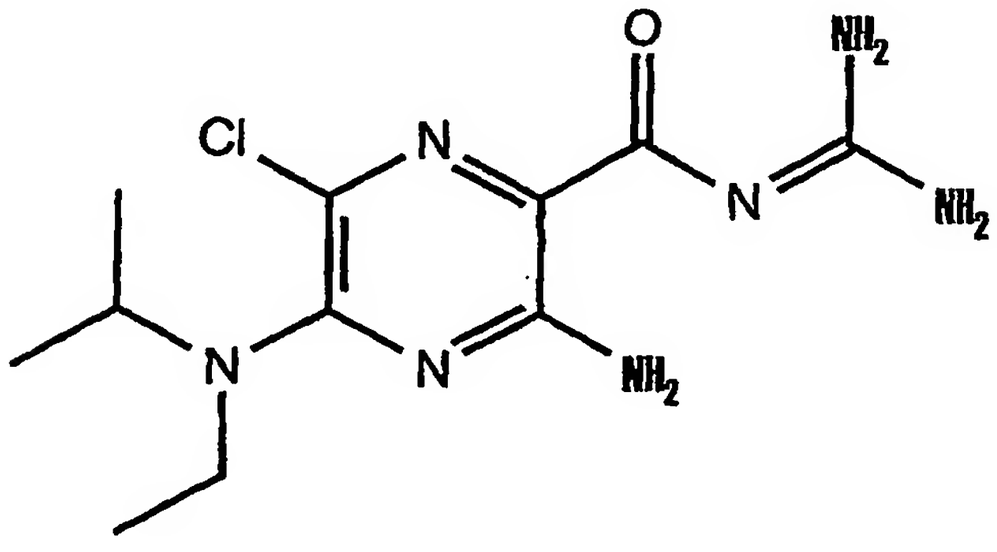
5-(N,N-Dimethyl)amiloride hydrochloride



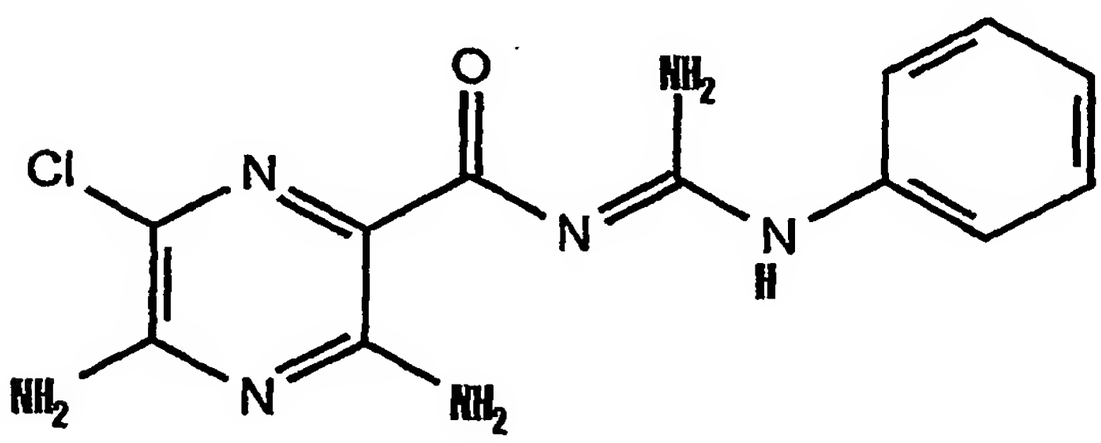
5-(N-methyl-N-isobutyl)amiloride comprising the structure



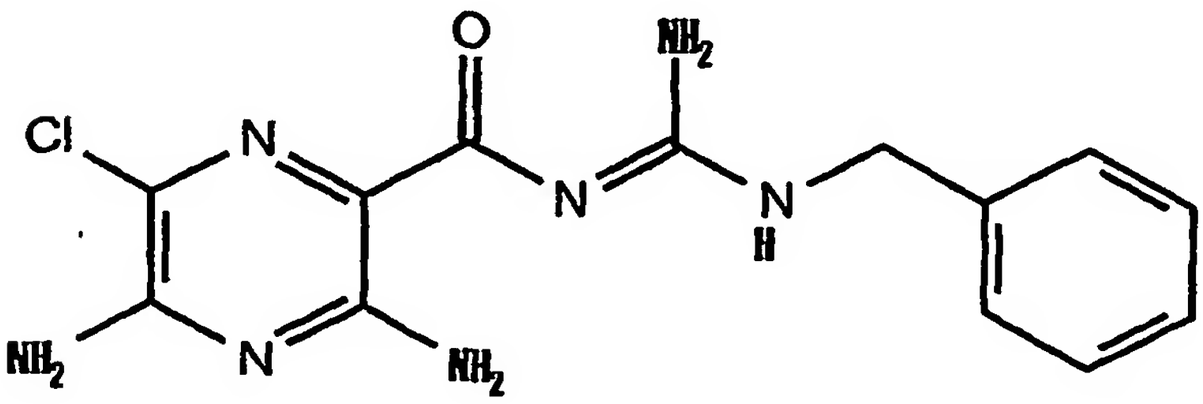
5-(N-ethyl-N-isopropyl)amiloride (herein referred to as EIPA), comprising the structure



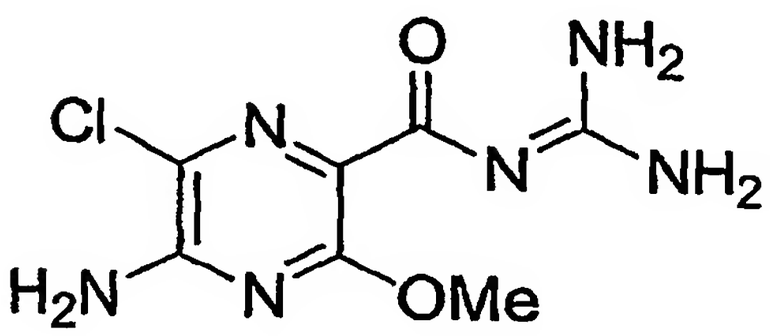
N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine, comprising the structure



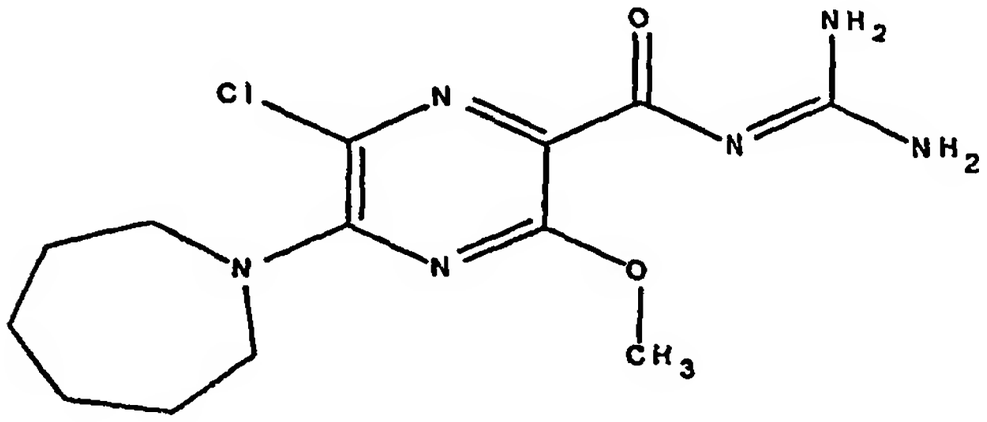
N-Benzyl –N'-(3,5-diamino-6-chloro-pyrzine-2-carbonyl)-guanidine, comprising the structure



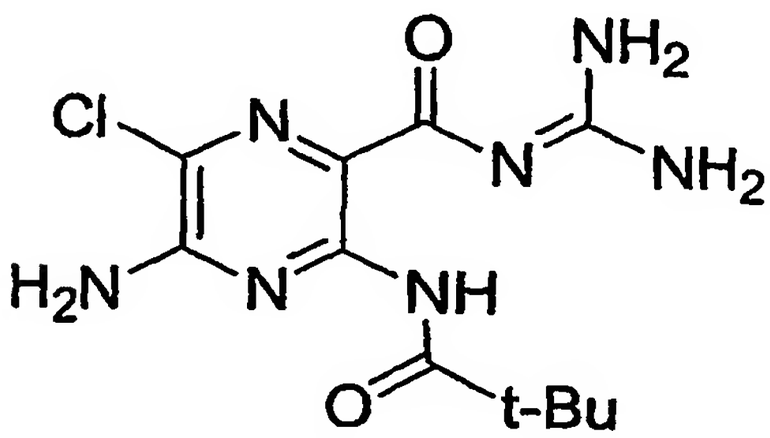
3-methoxy amiloride comprising the structure



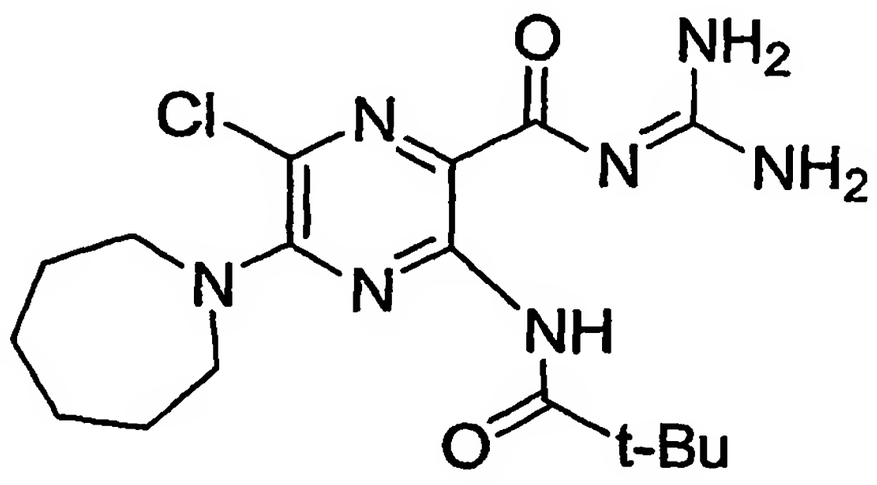
3-methoxy-5-(N,N-Hexamethylene)-amiloride comprising the structure



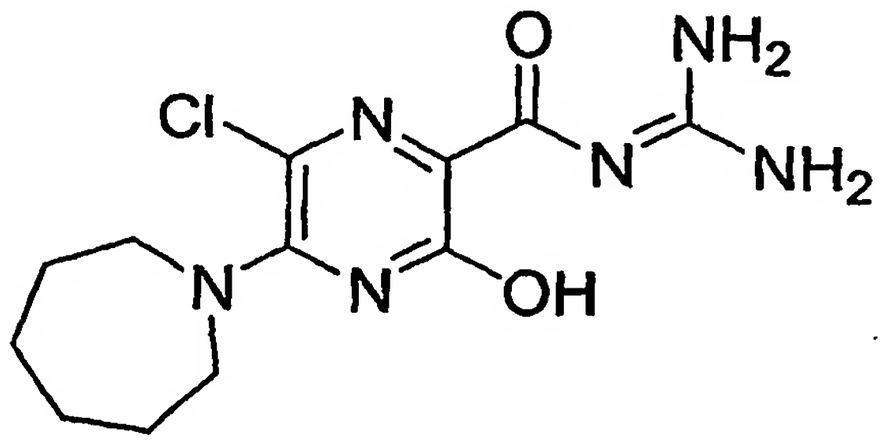
3-(N-2,2 -dimethyl propanal)amiloride comprising the structure



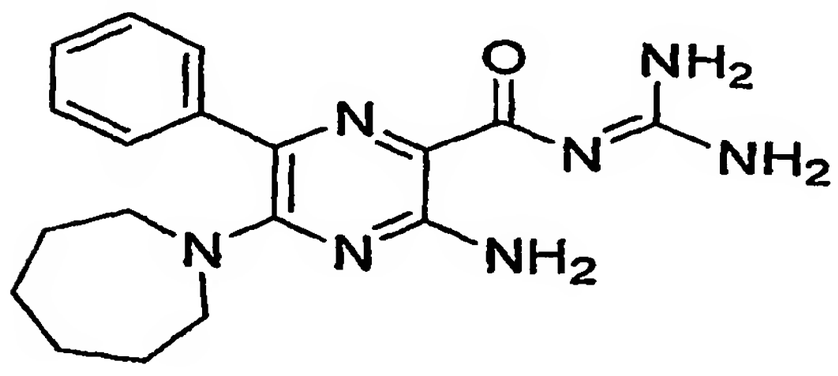
3-(N-2,2 -dimethyl propanal)-5-N-hexamethylene amiloride comprising the structure



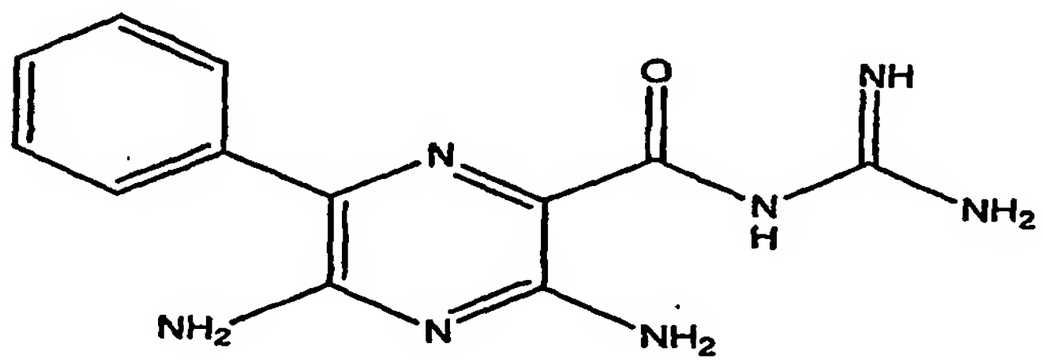
3-hydroxy-5-hexamethyleneimino-amiloride comprising the structure



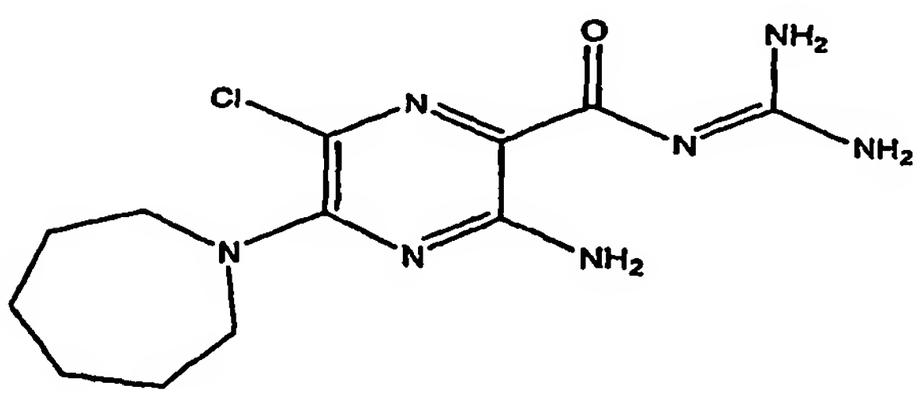
Hexamethyleneimino-6-phenyl-2-pyraxinecarboxamide comprising the structure



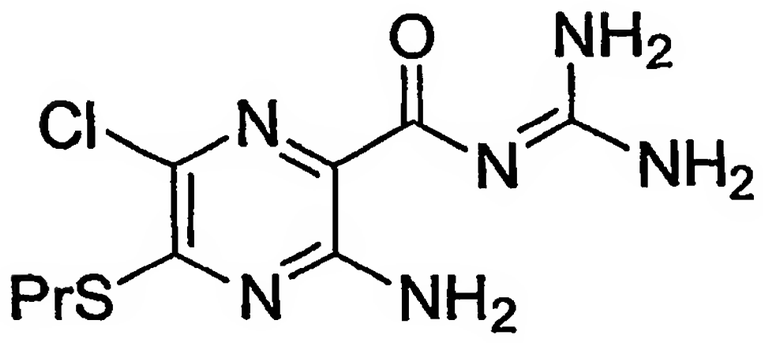
N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide comprising the structure



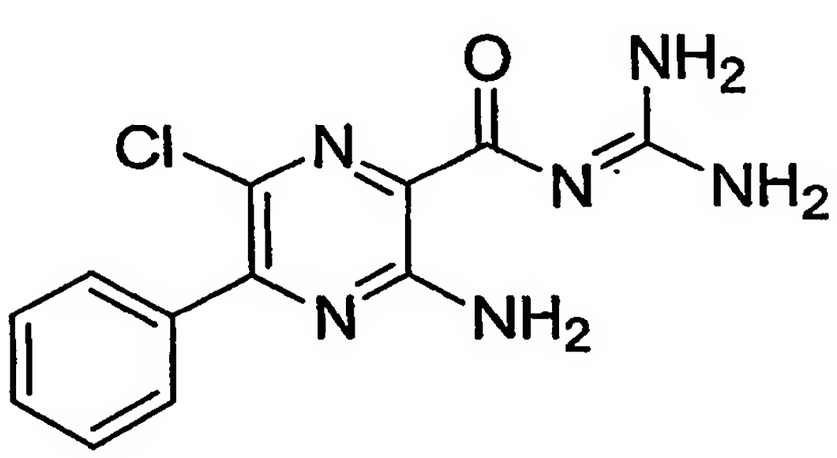
5-(N,N-hexamethylene)amiloride comprising the structure



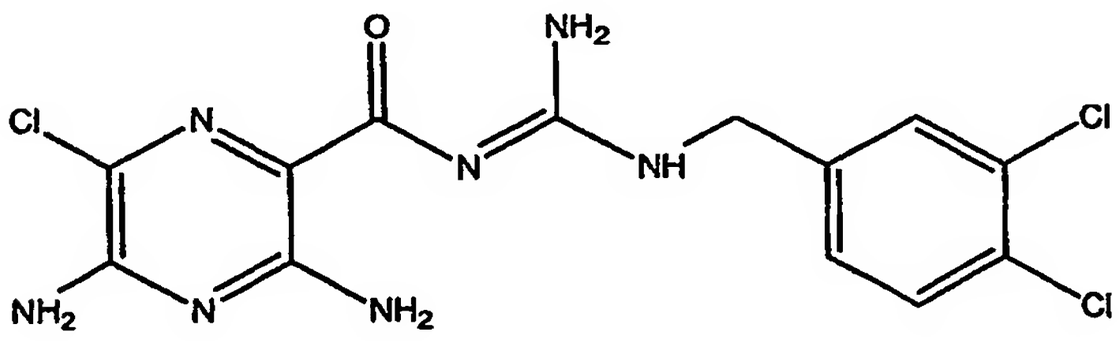
5-propyl-sulfide amiloride comprising the structure



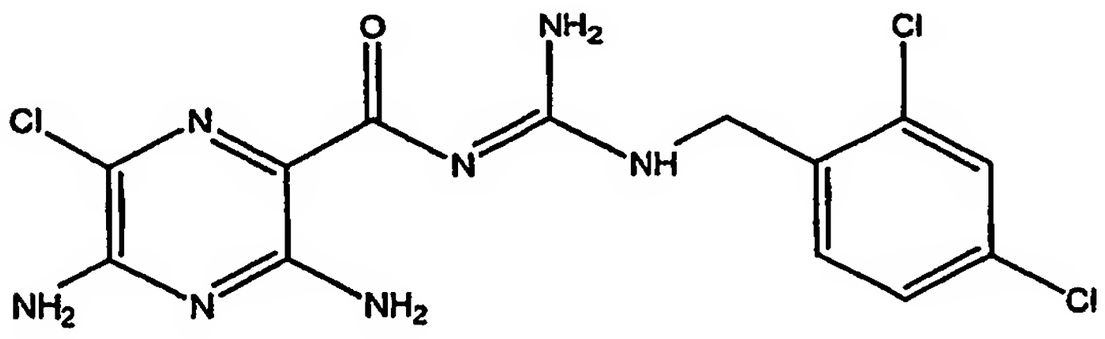
N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide comprising the structure



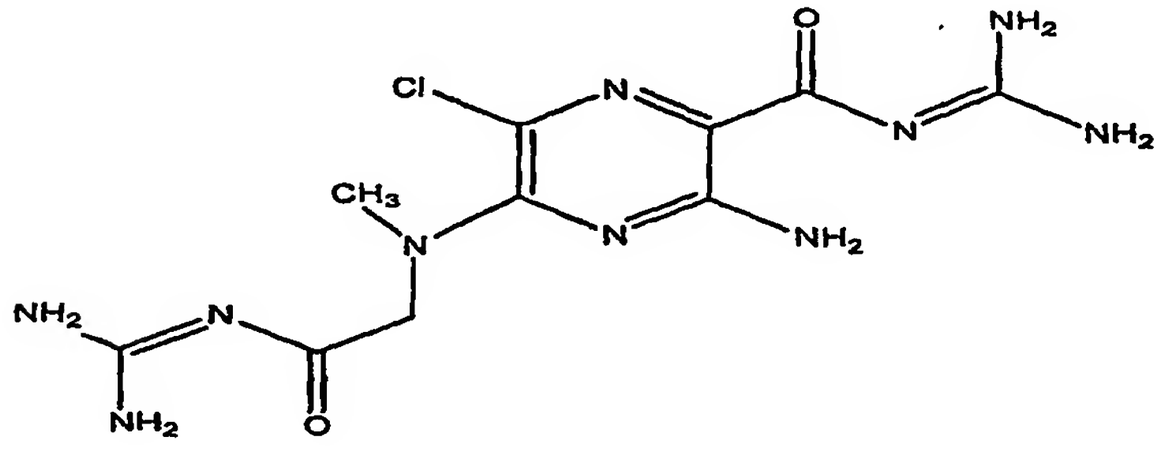
3'4 DichloroBenzamil comprising the structure



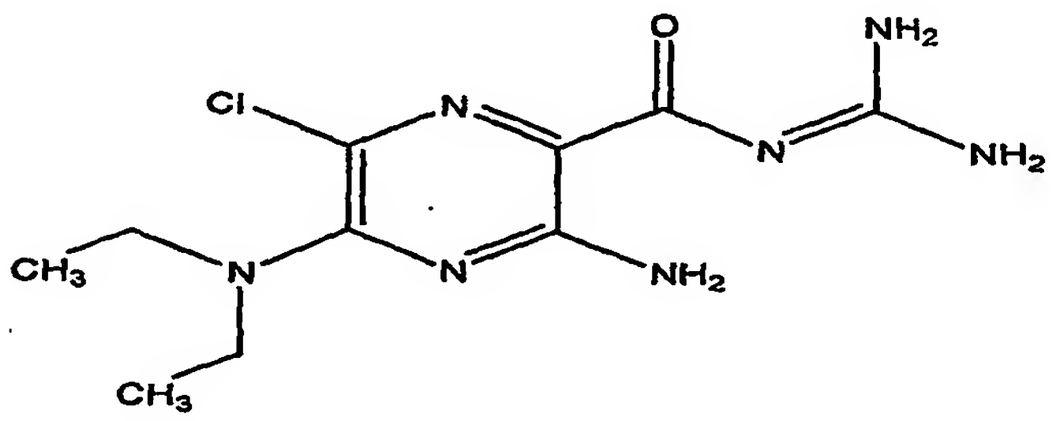
2'4 DichloroBenzamil HCl comprising the structure



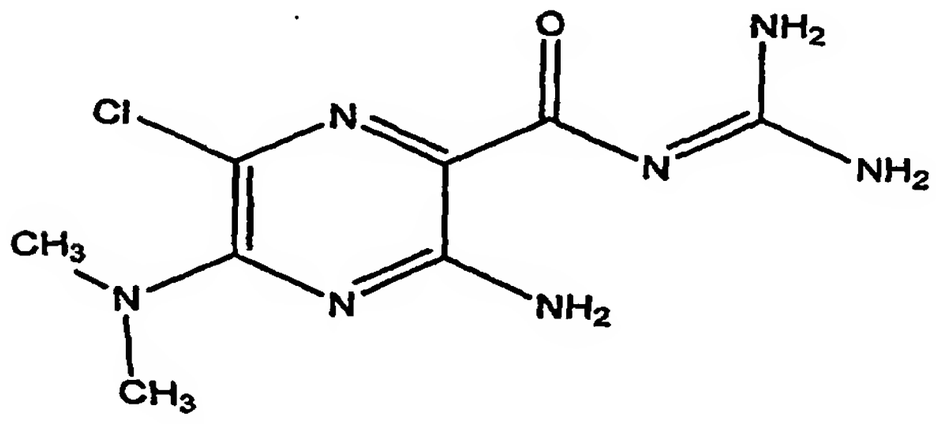
5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride comprising the structure



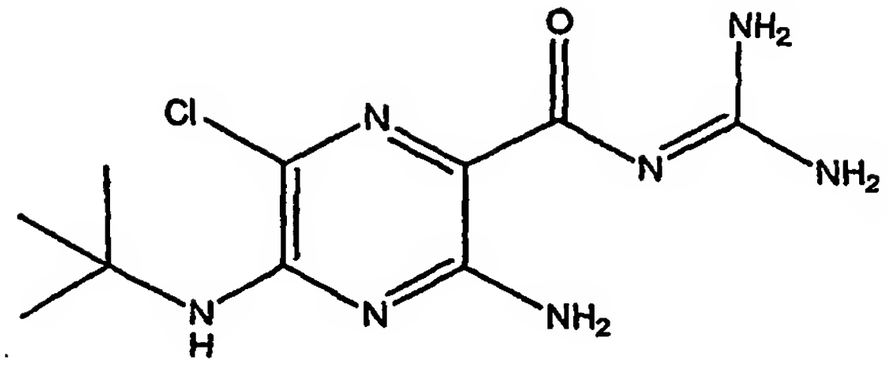
5-(N,N-Diethyl)amiloride hydrochloride comprising the structure



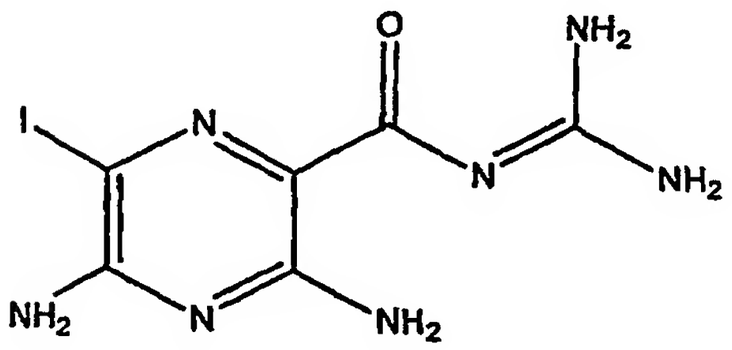
5-(N,N-Dimethyl)amiloride hydrochloride comprising the structure



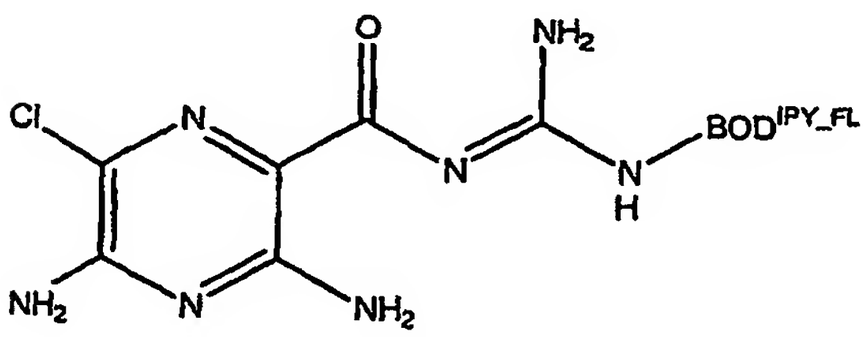
5-tert-butylamino-amiloride comprising the structure



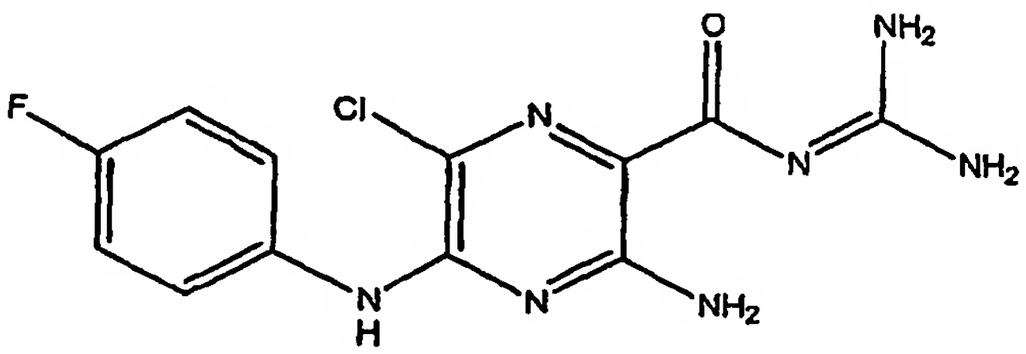
6-Iodoamiloride comprising the structure



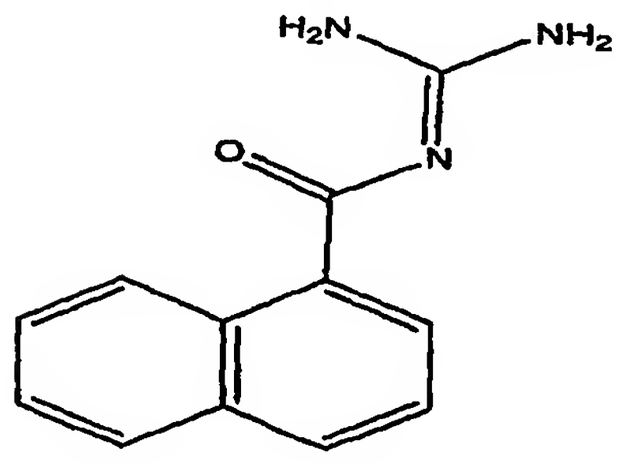
Bodipy-FL Amiloride comprising the structure



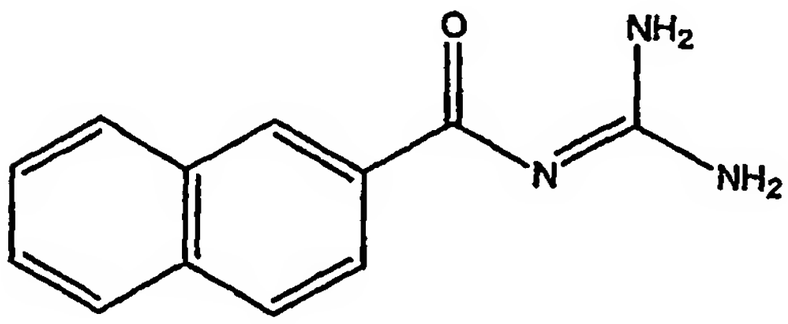
5-(4-fluorophenyl)amiloride comprising the structure



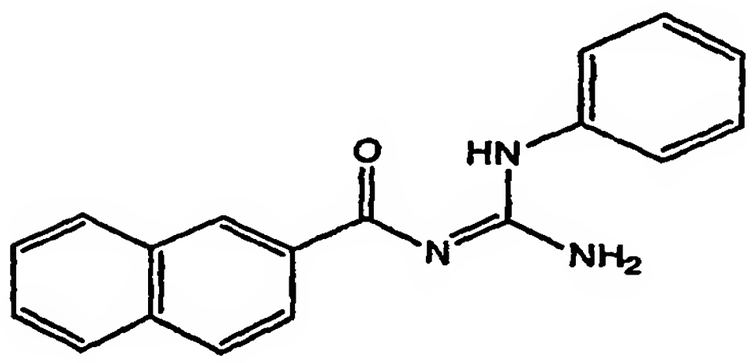
1-napthoylguanidine comprising the structure



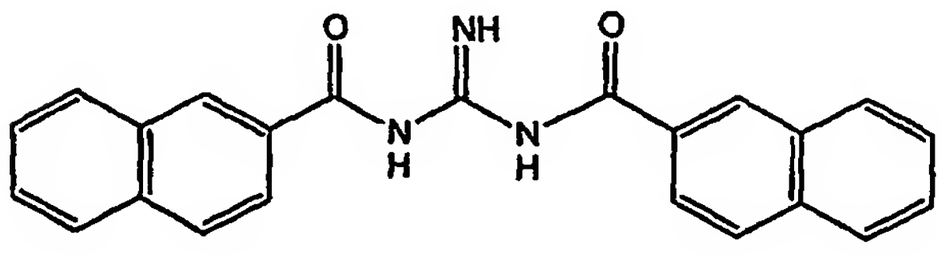
2-napthoylguanidine comprising the structure



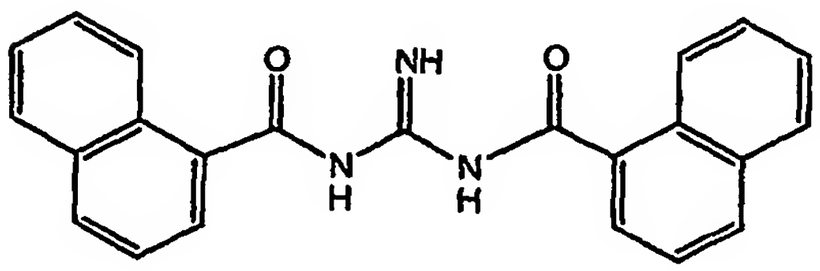
N-(2-napthoyl)-N'-phenylguanidine comprising the structure



N,N'-bis(2-napthoyl)guanidine comprising the structure



N,N'-bis(1-napthoyl)guanidine comprising the structure



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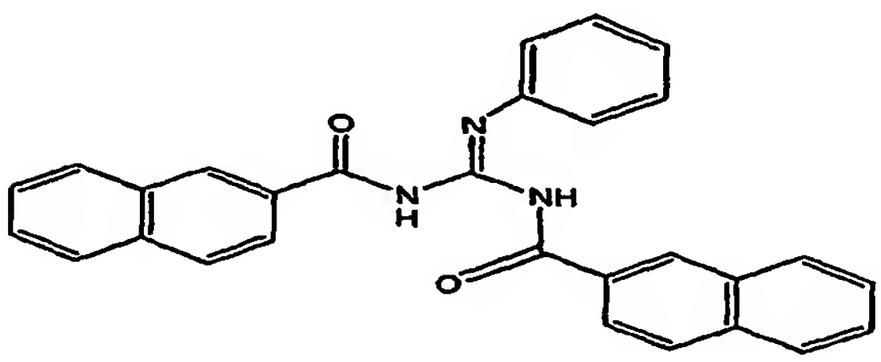
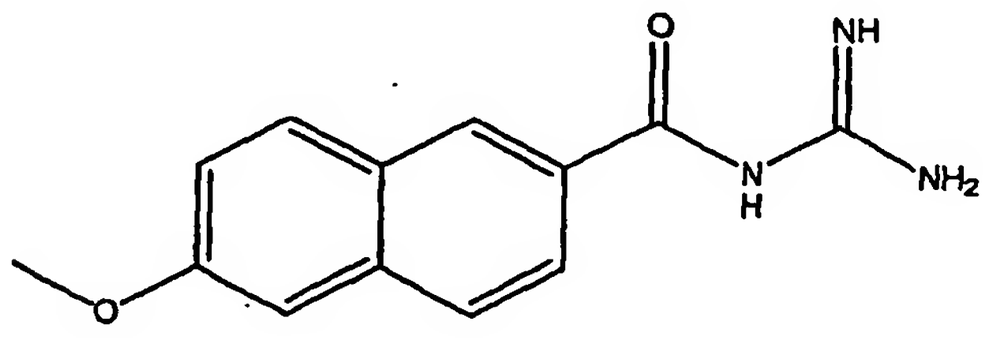
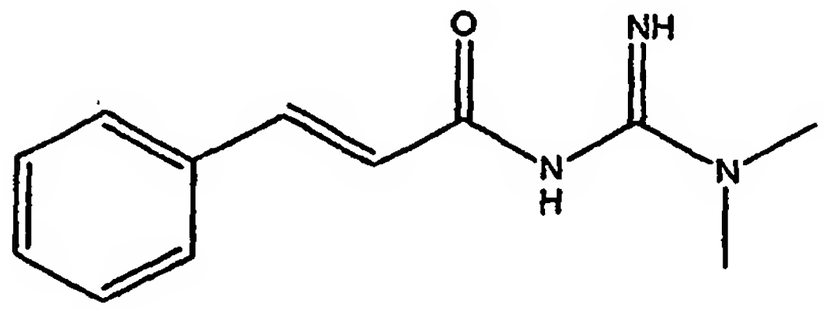
N,N'-bis(2-napthoyl)-N''-phenylguanidine comprising the structure



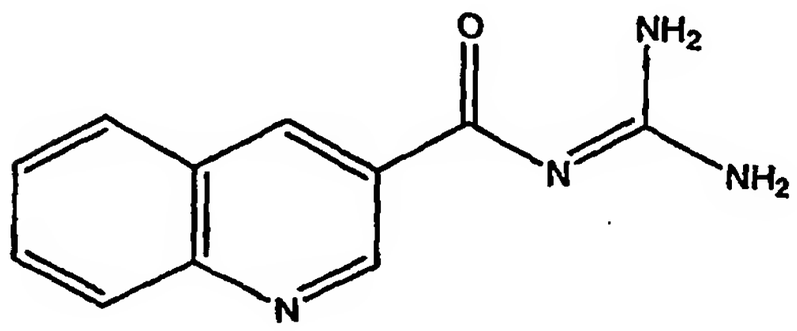
6-methoxy-2-naphthoylguanidine comprising the structure



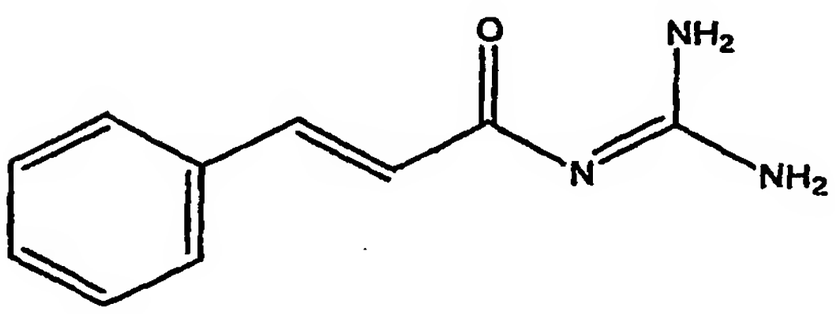
N-Cinnamoyl-N',N'-dimethylguanidine comprising the structure



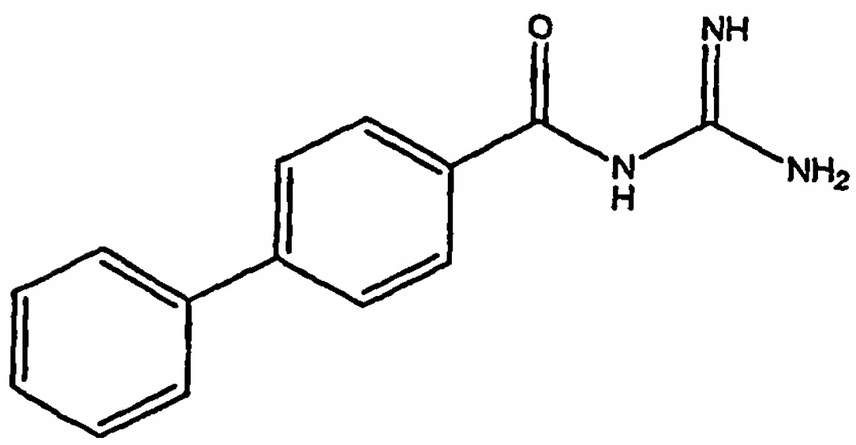
3-quinolinoylguanidine comprising the structure



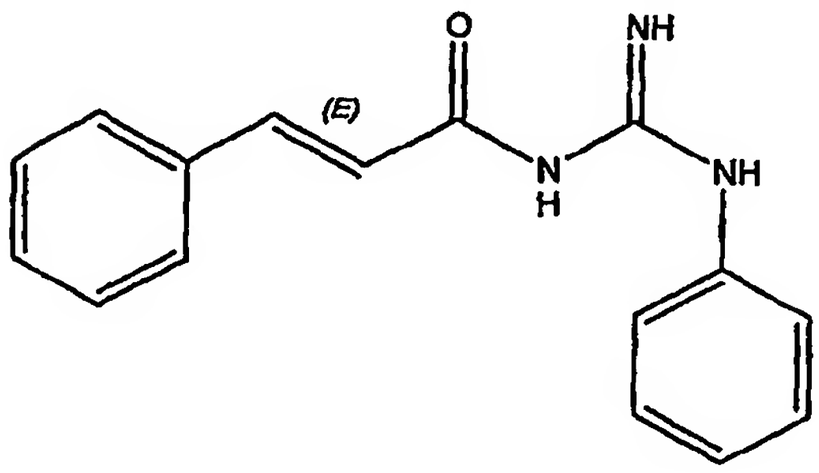
cinnamoylguanidine comprising the structure



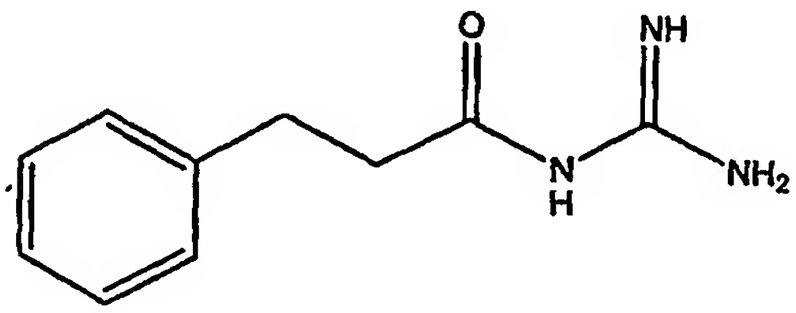
4-phenylbenzoylguanidine comprising the structure



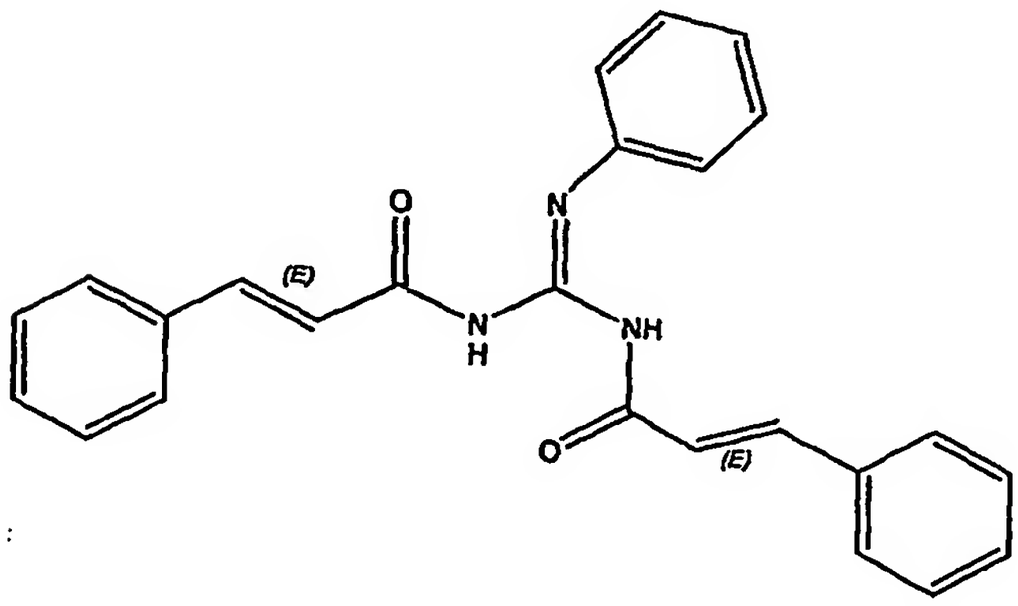
N-(cinnamoyl)-N'phenylguanidine comprising the structure



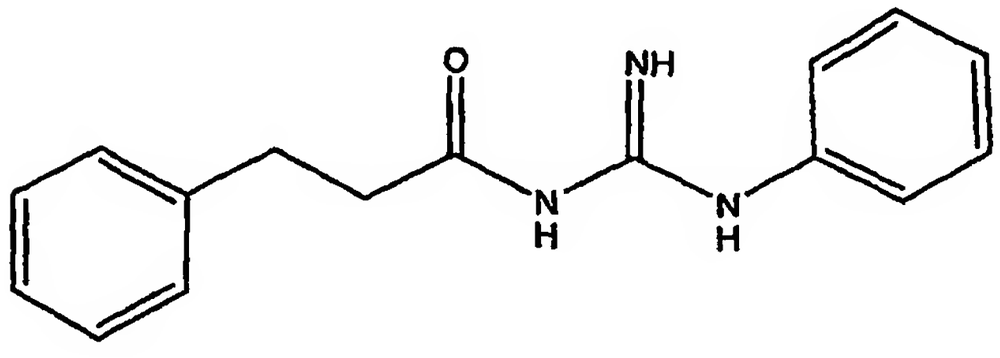
(3-phenylpropanoyl)guanidine comprising the structure



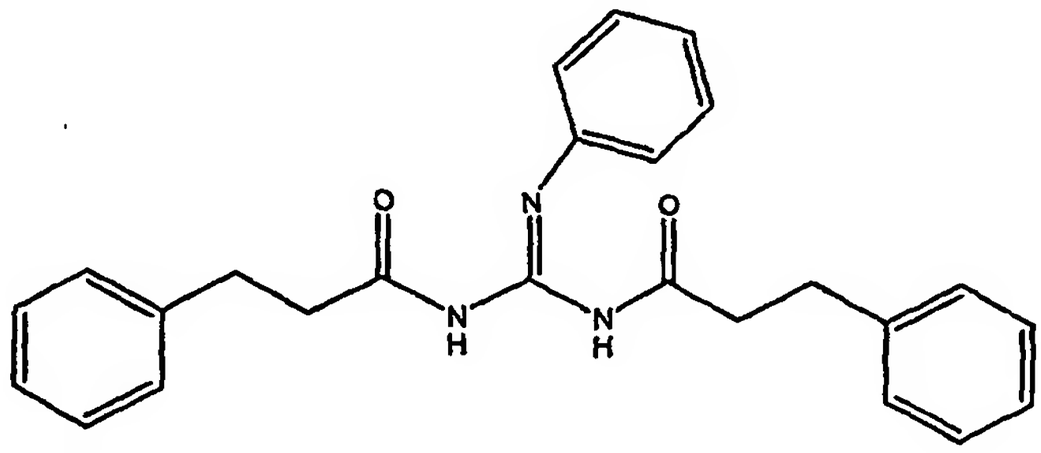
N,N'-bis-(cinnamoyl)-N"-phenylguanidine comprising the structure



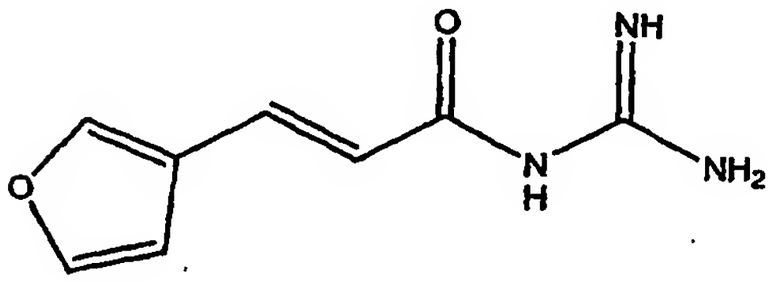
N-(3-phenylpropanoyl)-N'-phenylguanidine comprising the structure



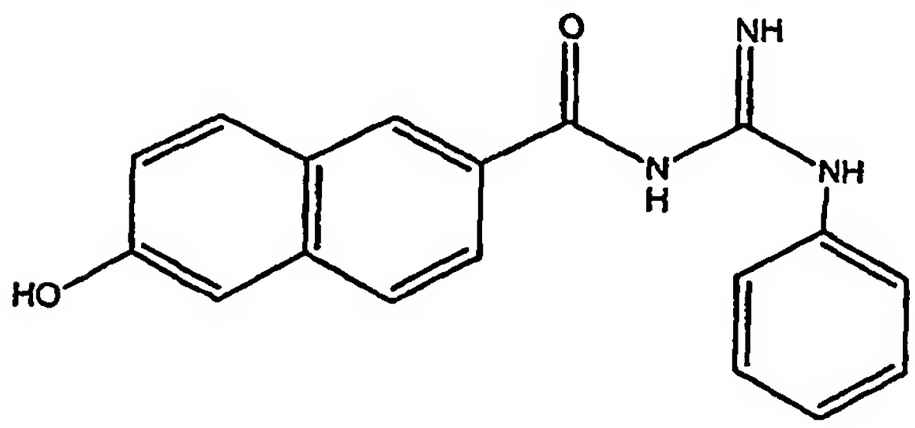
N,N'-bis(3phenylpropanoyl)-N"-phenylguanidine comprising the structure



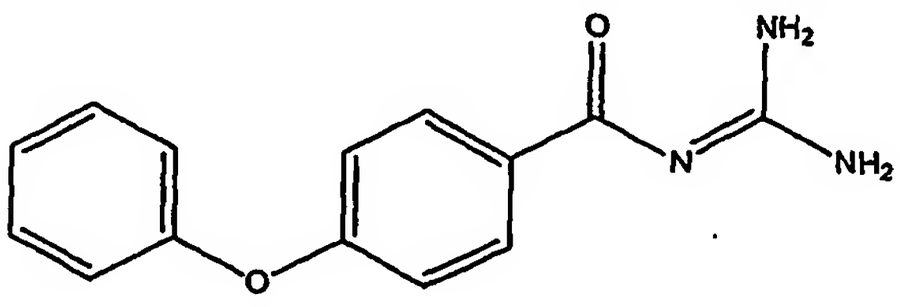
trans-3-furanacryoylguanidine comprising the structure



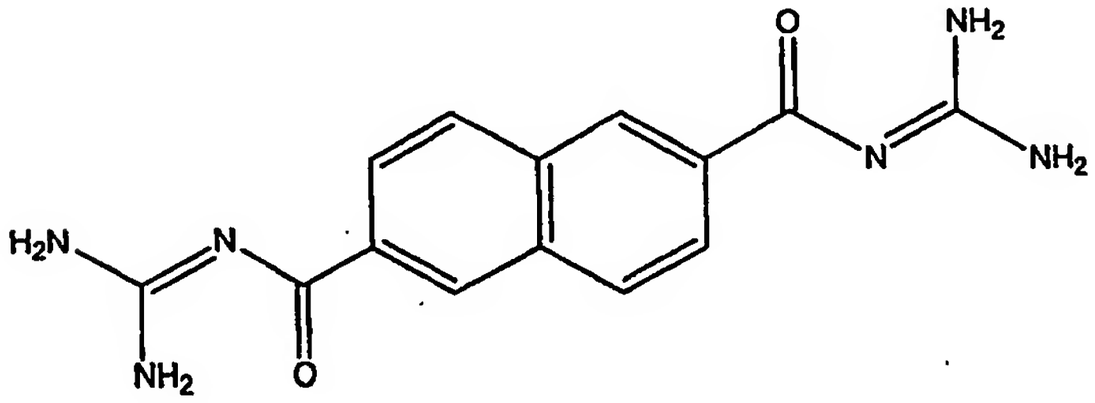
N-(6-Hydroxy-2-napthoyl)-N'-phenylguanidine comprising the structure



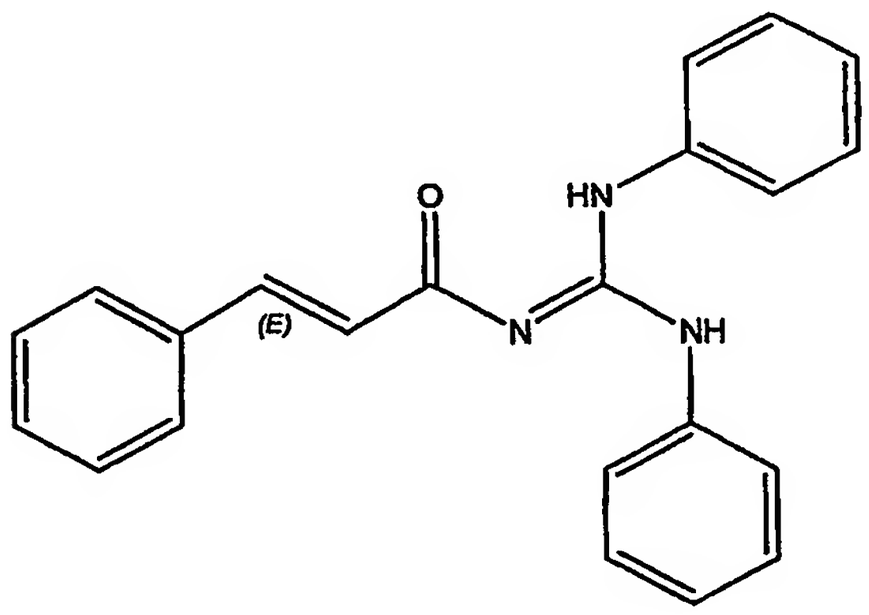
(4-Phenoxybenzoyl)guanidine comprising the structure



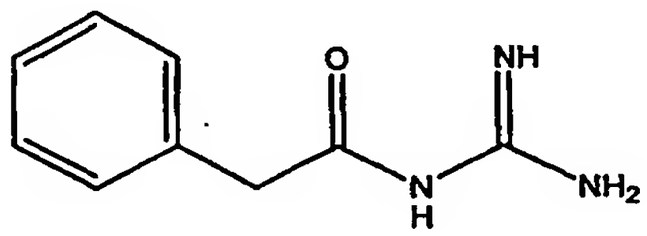
N,N'-Bis(amidino)napthalene-2,6-dicarboxamide comprising the structure



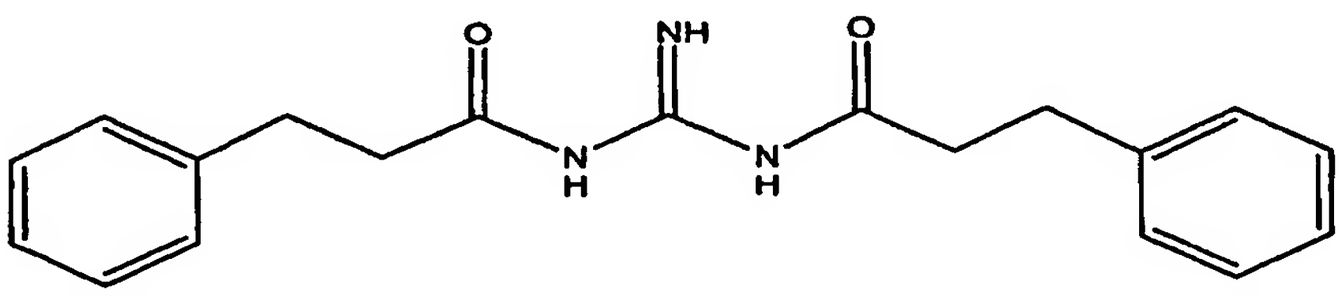
N''-Cinnamoyl-N,N'-diphenylguanidine comprising the structure



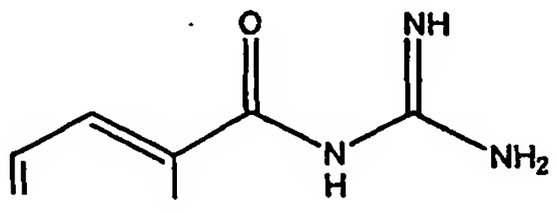
(Phenylacetyl)guanidine comprising the structure



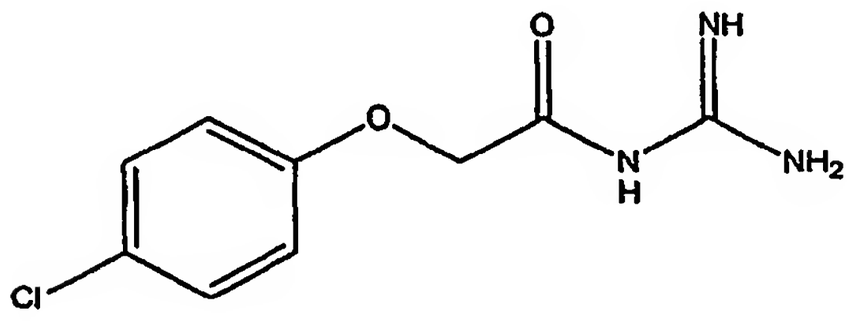
N,N'-Bis(3-phenylpropanoyl)guanidine comprising the structure



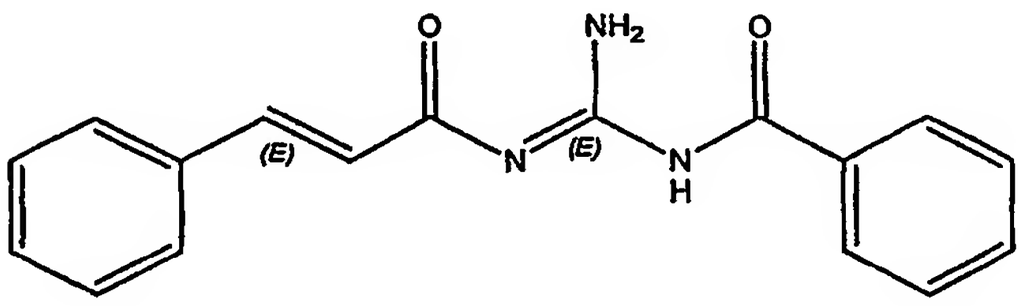
Benzyoylguanidine comprising the structure



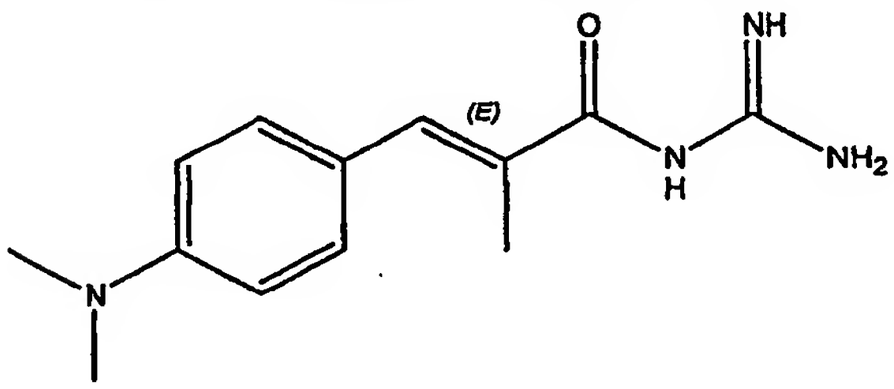
(4-Chlorophenoxy-acetyl]guanidine comprising the structure



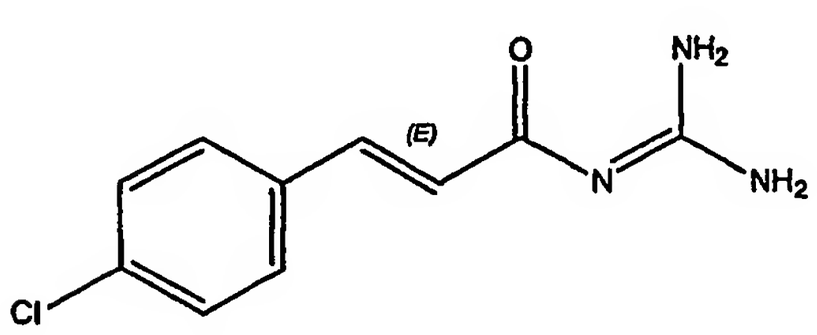
N-Benzoyl-N'-cinnamoylguanidine comprising the structure



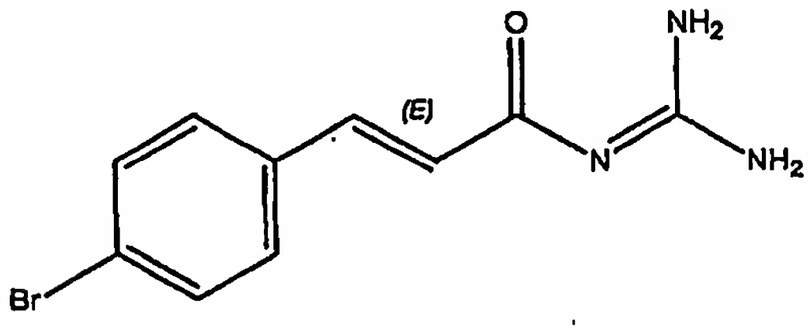
[(E)-3-(4-Dimethylaminophenyl)-2-methylacryloyl]guanidine comprising the structure



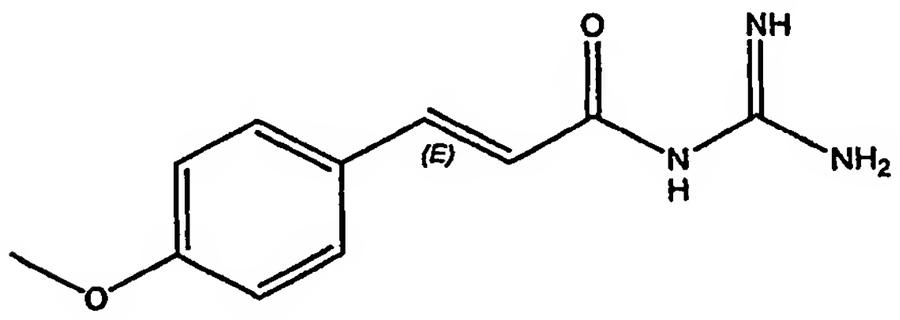
(4-Chlorocinnamoyl)guanidine comprising the structure



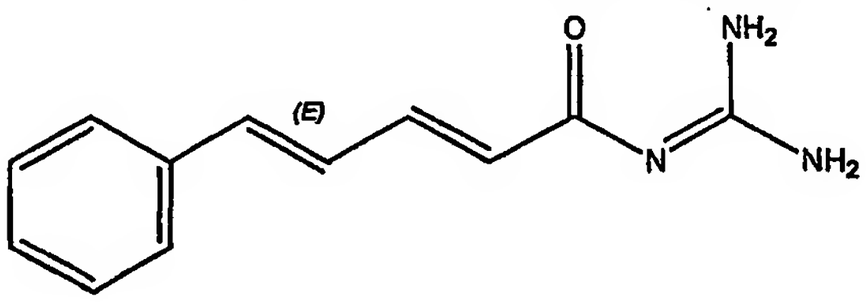
(4-Bromocinnamoyl)guanidine comprising the structure



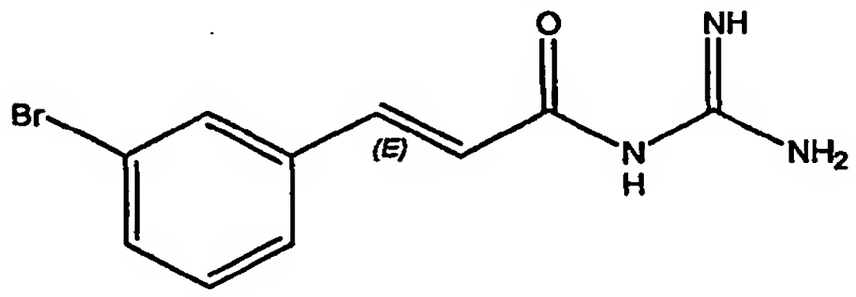
(4-Methoxycinnamoyl)guanidine comprising the structure



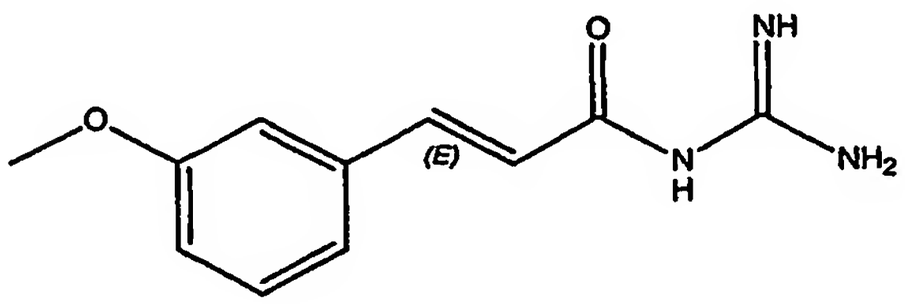
(5-Phenyl-penta-2,4-dienoyl)guanidine comprising the structure



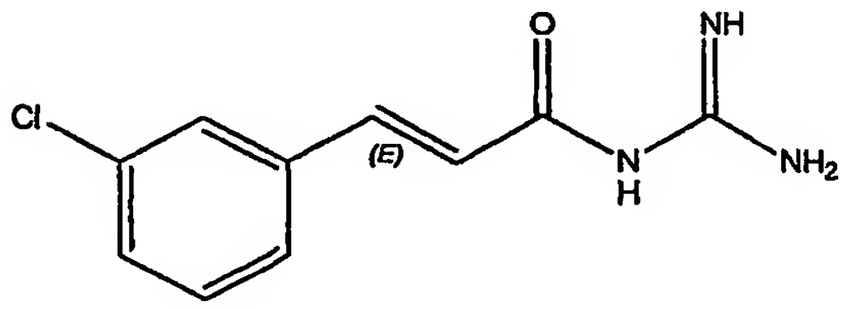
(3-Bromocinnamoyl)guanidine comprising the structure



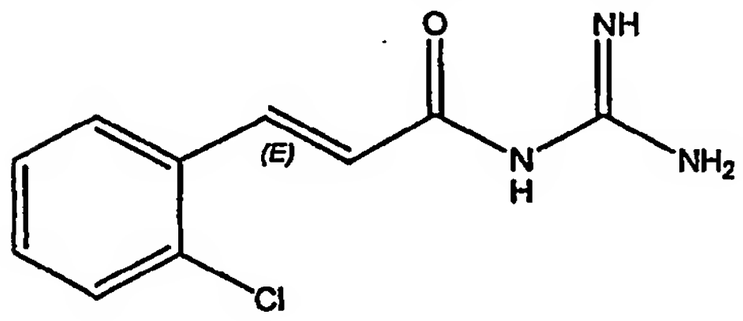
(3-Methoxycinnamoyl)guanidine comprising the structure



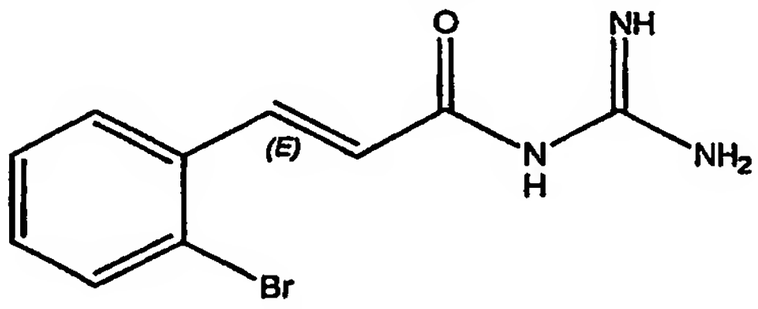
(3-Chlorocinnamoyl)guanidine comprising the structure



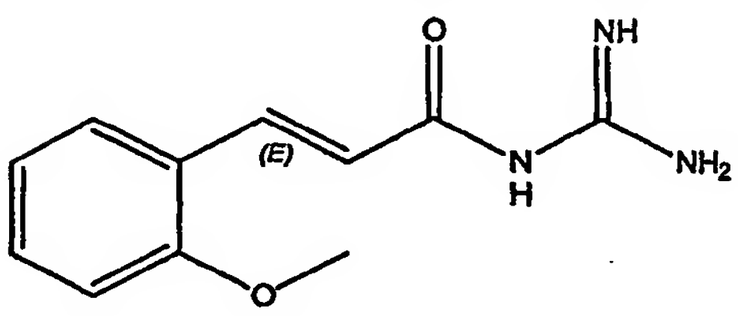
(2-Chlorocinnamoyl)guanidine comprising the structure



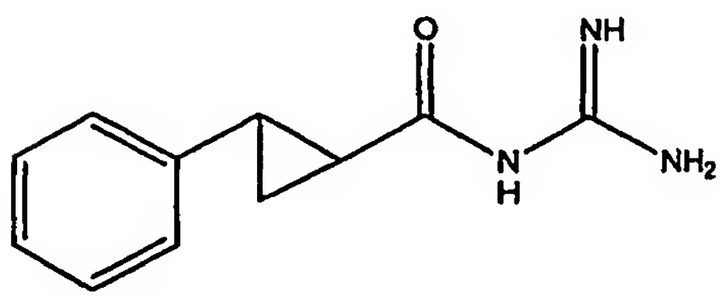
(2-Bromocinnamoyl)guanidine comprising the structure



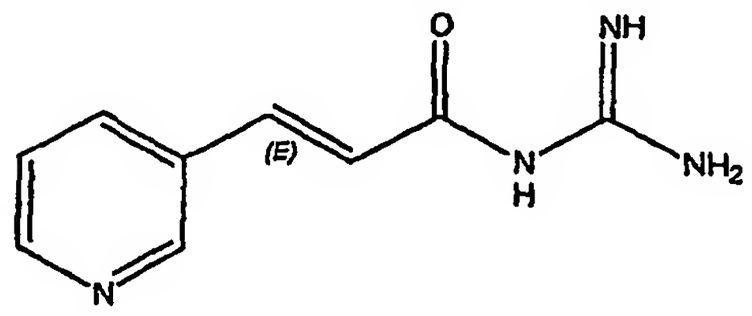
(2-Methoxycinnamoyl)guanidine comprising the structure



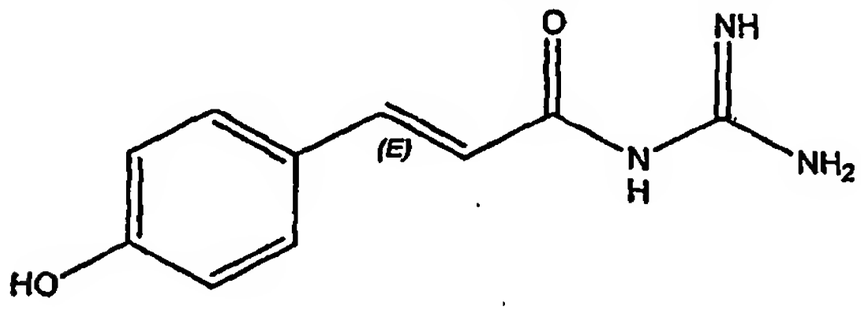
(trans-2-Phenylcyclopropanecarbonyl)guanidine comprising the structure



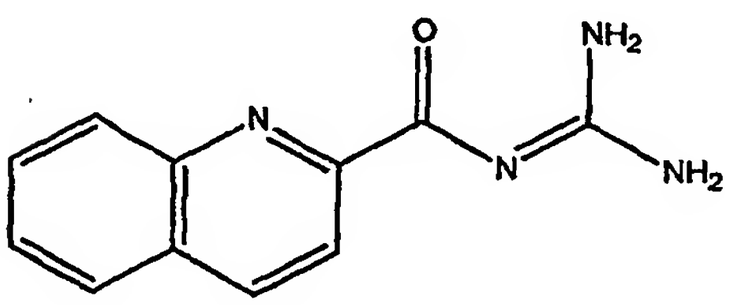
[3-(3-Pyridyl)acryloyl]guanidine comprising the structure



(4-Hydroxycinnamoyl)guanidine comprising the structure



(Quinoline-2-carbonyl)guanidine comprising the structure



or as provided in the following list:

N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine (also referred to as phenamil) (Sigma),

N-Benzyl-N'-(3,5-diamino-6-chloro-pyrazine-2-carbonyl)-guanidine (also referred to as Benzamil) (Sigma),

3'4 DichloroBenzamil,

2'4 DichloroBenzamil HCl (BioMol CA-204),

5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride,

5-(N-Methyl-N-isobutyl)amiloride (Sigma),

5-(N-Ethyl-N-isopropyl)amiloride (Sigma),

5-(N,N-Dimethyl)amiloride hydrochloride (Sigma),

5-(N,N-hexamethylene)amiloride (Sigma),

5-(N,N-Diethyl)amiloride hydrochloride (Molecular Probes),

6-Iodoamiloride (Molecular Probes),

Bodipy-FL Amiloride (Molecular Probes),

3-hydroxy-5-hexamethyleneimino-amiloride,

5-(4-fluorophenyl)amiloride,

5-tert-butylamino-amiloride (Chem. Pharm. Bull., 1997 **45,** 1282-1286),

N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide,

3-methoxy –HMA,

3-(N-2,2-dimethyl propanal)amiloride,

3-(N-2,2 –dimethyl propanal)-5-N-hexamethylene amiloride

3-methoxy-amiloride,

5-propyl-sulfide amiloride,

hexamethyleneimino-6-phenyl-2-pyrazinecarboximide,

N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide,

1-napthoylguanidine,

2-napthoylguanidine (Chem. Pharm. Bull., 1997 **45,** 1282-1286),

N-(2-napthoyl)-N'-phenylguanidine,

N,N'-bis(2-napthoyl)guanidine,

N,N'-bis(1-napthoyl)guanidine,

N,N'-bis(2-napthoyl)-N"-phenylguanidine ,

6-methoxy-2-naphthoylguanidine (Chem. Pharm. Bull., 1997 **45,** 1282-1286),

3-quinolinoylguanidine (Chem. Pharm. Bull., 1997 **45,** 1282-1286),

cinnamoylguanidine (J. Amer. Chem. Soc., 1954, **76,** 574-576),

4-phenylbenzoylguanidine,

N-(cinnamoyl)-N'phenylguanidine (WO 84/00875),

(3-phenylpropanoyl)guanidine (WO 84/00875),

N,N'-bis-(cinnamoyl)-N"-phenylguanidine,

N-(3-phenylpropanoyl)-N'-phenylguanidine (WO 84/00875),

N,N'-bis(3phenylpropanoyl)-N"-phenylguanidine,

trans-3-furanacryoylguanidine,

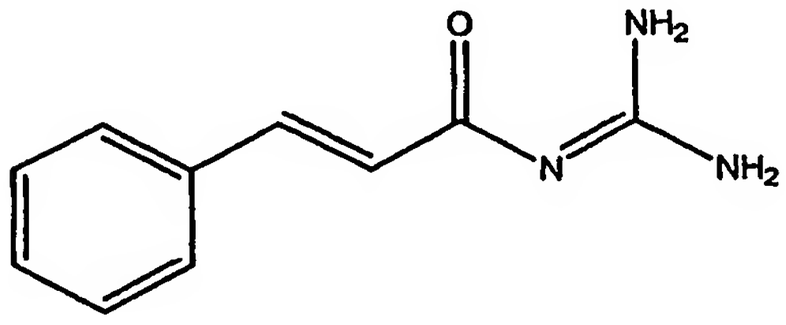
N-(6-Hydroxy-2-napthoyl)-N'-phenylguanidine,

(4-Phenoxybenzoyl)guanidine,

N,N'-Bis(amidino)napthalene-2,6-dicarboxamide,

N''-Cinnamoyl-N,N'-diphenylguanidine,
(Phenylacetyl)guanidine,
N,N'-Bis(3-phenylpropanoyl)guanidine,
Benzyoylguanidine,
(4-Chlorophenoxy-acetyl]guanidine,
N-Benzoyl-N'-cinnamoylguanidine,
[(E)-3-(4-Dimethylaminophenyl)-2-methylacryloyl]guanidine,
(4-Chlorocinnamoyl)guanidine,
(4-Bromocinnamoyl)guanidine,
(4-Methoxycinnamoyl)guanidine,
(5-Phenyl-penta-2,4-dienoyl)guanidine,
(3-Bromocinnamoyl)guanidine,
(3-Methoxycinnamoyl)guanidine,
(3-Chlorocinnamoyl)guanidine,
(2-Chlorocinnamoyl)guanidine,
(2-Bromocinnamoyl)guanidine,
(2-Methoxycinnamoyl)guanidine,
(trans-2-Phenylcyclopropanecarbonyl)guanidine,
[3-(3-Pyridyl)acryloyl]guanidine,
(4-Hydroxycinnamoyl)guanidine,
(Quinoline-2-carbonyl)guanidine

One of the preferred compounds of the invention comprises the structure

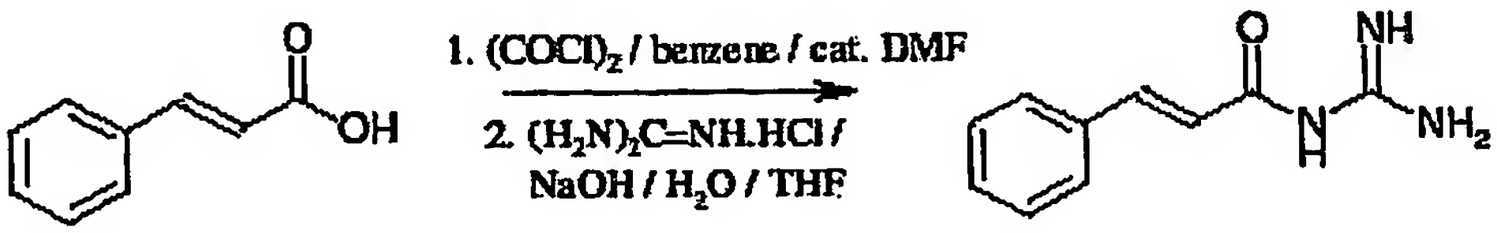


cinnamoylguanidine (Bit036).

EXAMPLES

Example 1.

Synthesis of Cinnamoylguanidine from Cinnamic acid Cinnamoyl chloride

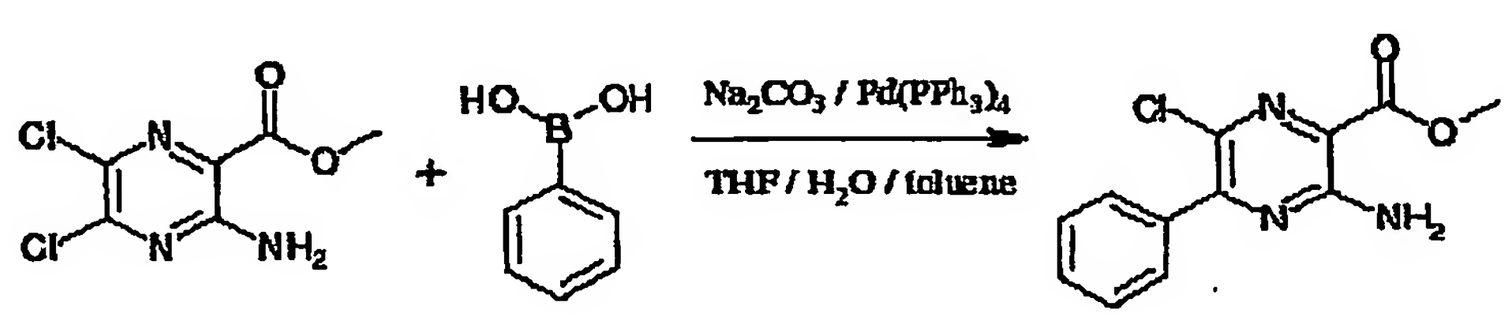


To a solution of *trans*-cinnamic acid (1.50 g, 10.12 mmol) in dry benzene (30mL) containing a drop of *N,N*-dimethylformamide was added oxalyl chloride (5.14 g, 40.5 mmol) causing the solution to effervesce. After refluxing for 2 h, the solution was evaporated to dryness under reduced pressure. The resulting solid was dissolved in dry tetrahydrofuran (20mL) and added slowly to a solution of guanidine hydrochloride in 2M aqueous sodium hydroxide (25mL). The reaction was stirred at room temperature for 1h then extracted with ethyl acetate (3x50mL). The combined extracts were dried over magnesium sulfate and evaporated to give an orange oil. The crude product was purified by column chromatography. Elution with 10% to 20% methanol in dichloromethane gave *Cinnamoylguanidine* as a cream solid (0.829 g, 43%).

Example 2

Synthesis of N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide

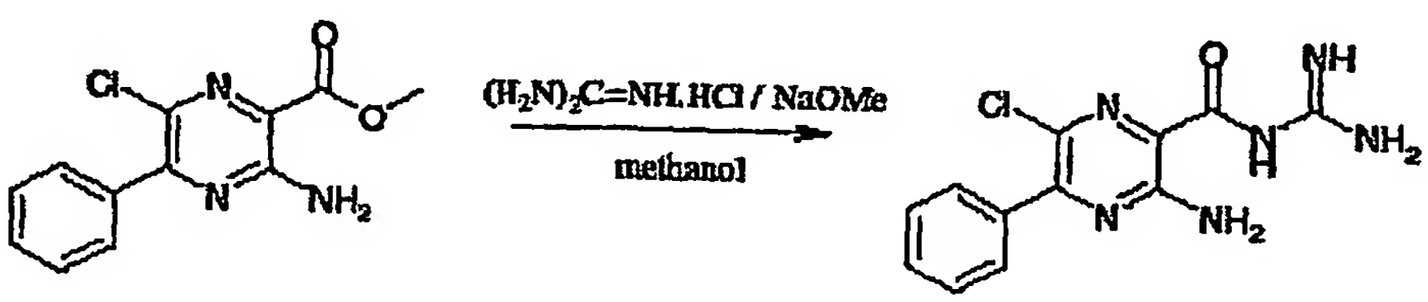
Part 1



To a solution of methyl 3-amino-5,6-dichloro-2-pyrazinecarboxylate (0.444 g, 2.0 mmol) in tetrahydrofuran (5 mL) / water (10 mL) / toluene (20 mL) was added phenyl boronic acid (0.536 g, 4.4 mmol), sodium carbonate (0.699 g, 6.6 mmol) and tetrakis(triphenylphosphine)- palladium(0) (0.116 g, 0.10 mmol). The reaction was evacuated and purged with nitrogen several times before being refluxed for 6 h. The organic layer was separated and the aqueous layer extracted with toluene (3 x 20 mL).

The combined organic extracts were dried over magnesium sulfate, filtered and evaporated under reduced pressure to give *methyl 3-amino-6-chloro-5-phenyl-2-pyrazinecarboxylate* as a yellow solid (0.43 g, 82%).

Part 2

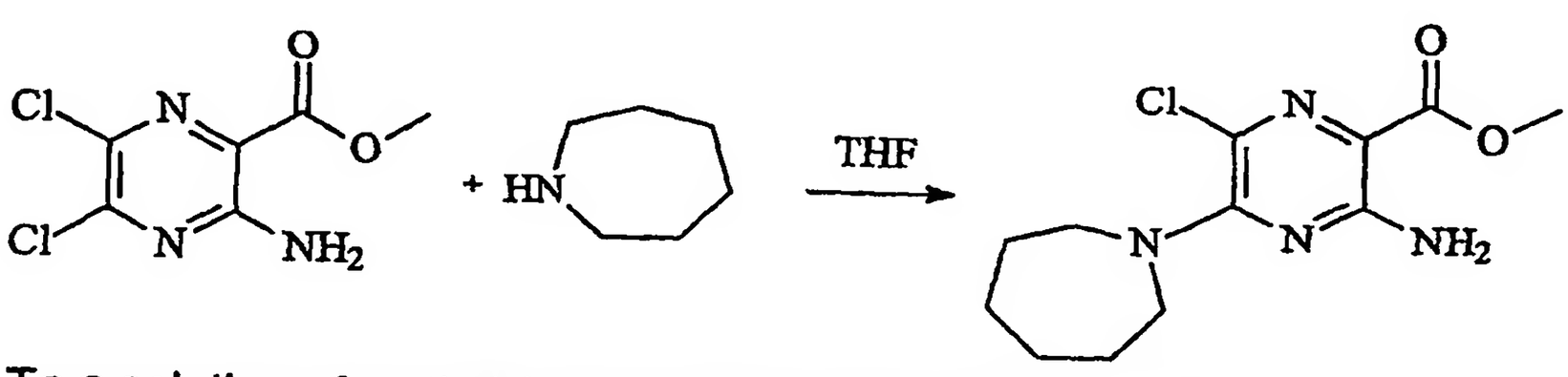


To a solution of sodium (0.040 g, 1.74 mmol) dissolved in methanol (5 mL) was added guanidine hydrochloride (0.258 g, 2.70 mmol) and the mixture refluxed for 30 min after which it was filtered. To the filtrate was added methyl 3-amino-6-chloro-5-phenyl-2-pyrazinecarboxylate (0.264 g, 1.0 mmol) in *N,N*-dimethylformamide (5 mL) and the solution heated at 75oC for 12 h. The solvent was removed under reduced pressure and the residue chromatographed on silica gel eluting with 1% triethylamine / 5% methanol / dichloromethane. The resulting solid was suspended in chloroform, filtered and dried under high vacuum to give *N-Amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide* as a yellow solid (0.04 g, 14%).

Example 3.

Synthesis of hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide

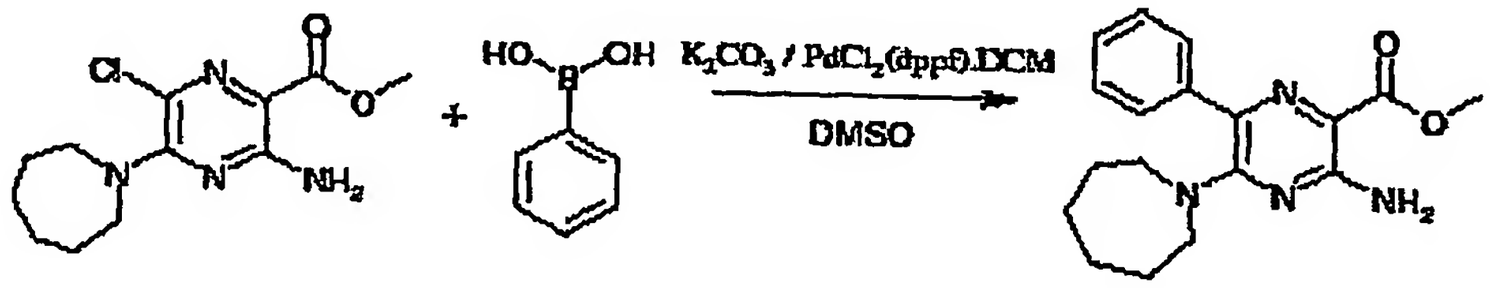
Part 1



To a solution of methyl 3-amino-5,6-dichloro-2-pyrazinecarboxylate (1.11 g, 5.0 mmol) in tetrahydrofuran (50 mL) was added hexamethyleneimine (1.49 g, 15.0 mmol) and the reaction was refluxed for 1 h. The reaction was allowed to cool and the solid hexamethyleneimine hydrochloride removed by filtration. The filtrate was evaporated and the residue chromatographed over silica gel. Elution with dichloromethane gave

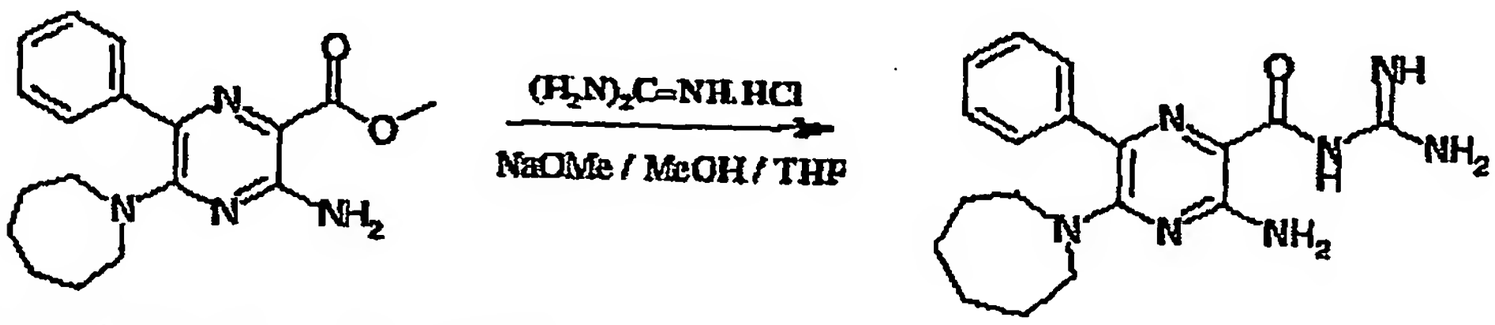
methyl 3-amino-6-chloro-5-hexamethyleneimino-2-pyrazinecarboxylate as an off-white solid (1.20 g, 85%).

Part 2



To a solution of methyl 3-amino-6-chloro-5-hexamethyleneimino-2-pyrazinecarboxylate (0.350g, 1.23 mmol) in dimethylsulfoxide (5 mL) was added phenyl boronic acid (0.166 g, 1.35 mmol), potassium carbonate (0.511 g, 3.70 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II)-dichloromethane complex (0.041 g, 0.05 mmol). The reaction was heated at 90oC for 16 h before being poured into water (50mL) and extracted with ethyl acetate (3 x 50mL). The combined extracts were dried over magnesium sulfate, filtered and evaporated to give a brown oil which was purified by chromatography on silica gel. Elution with dichloromethane followed by 10% ethyl acetate/dichloromethane gave *methyl 3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxylate* as a yellow solid (0.309 g, 77%).

Part 3.



To a solution of sodium (0.090 g, 6.17 mmol) dissolved in methanol (8 mL) was added guanidine hydrochloride (0.598 g, 6.26 mmol) and the mixture was refluxed for 30 min after which it was filtered. To the filtrate was added methyl 3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxylate (0.310 g, 0.95 mmol) in tetrahydrofuran (10 mL) and the solution refluxed for 72 h. The solvent was removed under reduced pressure and the residue chromatographed on silica gel. Elution with 5% methanol/dichloromethane gave *N-amidino-3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide* as a yellow solid (0.116 g, 35%).

Example 4. Peptide Synthesis

The C- terminal 40 amino acids of the M protein of the Dengue virus type 1 strain Singapore S275/90 (Fu et al 1992)

(ALRHPGFTVIALFLAHAIGTSITQKGIIFILLMLVTPSMA) was synthesised using the Fmoc method. The synthesis was done on a Symphony Peptide Synthesiser form Protein Technologies Inc (Tucson, Arizona) as used to give C-terminal amides, the coupling was done with HBTU and hydroxybenzotriazole in N-methylpyrrolidone. Each of the synthesis cycle used double coupling and a 4-fold excess of the amino acids. Temporary α-N Fmoc-protecting groups were removed using 20% piperidine in DMF.

Example 5. Channel Recordings

Lipid bilayer studies were performed as described elsewhere (Sunstrom, 1996; Miller, 1986). A lipid mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, Alabama) was used. The lipid mixture was painted onto an aperture of 150-200 µm in the wall of a 1 ml delrin cup. The aperture separates two chambers, cis and trans, both containing salt solutions at different concentrations. The cis chamber was connected to ground and the trans chamber to the input of an Axopatch 200 amplifier. Normally the cis chamber contained 500 mM KCl and the trans 50 mM KCl. The bilayer formation was monitored electrically by the amplitude of the current pulse generated by a current ramp. The potentials were measured in the trans chamber with respect to the cis. The protein was added to the cis chamber and stirred until channel activity was seen. The currents were filtered at 1000 Hz, digitized at 5000 Hz and stored on magnetic disk.

The dengue virus M protein C-terminal peptide (DMVC) was dissolved in 2,2,2-trifluorethanol (TFE) at 0.05mg/ml to 1 mg/ml. 10 µl of this was added to the cis chamber of the bilayer which was stirred. Channel activity was seen within 15-30 min.

Example 6. Hexamethylene amiloride (HMA) to inhibits ion channel activity of the dengue virus M protein C-terminal peptide.

Solutions of 50 mM HMA were prepared by first making a 500 mM solution in DMSO. This solution was further diluted to 50 mM HMA using 0.1 M HCl. 2 µl of the 50 mM HMA was added to the cis chamber after channel activity was seen. The cis chamber contained 1 ml of solution making the final concentration of HMA 100 µM.

Example 7. Plaque assay screen of anti-flavivirus compounds

Compounds can be screened for their ability to inhibit budding of various different flaviviruses by a number of well known assays, including the plaque assay. Suitable methods are described in standard texts, such as for example " Fundamental techniques in virology", ed. / by K. Habel and N.P. Salzman. N.Y., Academic Press, 1969 and Basic medical virology. / Balt., Williams & Wilkins, 1966 or Adolph, K.W. (ed) (1994) Molecular virology techniques". In: Methods in Molecular Genetics, Vol. 4. New York: Academic Press, both of which are incorporated in their entirety herein by reference.

Briefly the procedure is as follows:

Cells susceptible to infection with the flavivirus to be tested are plated into 6 well plates and grown to confluence. Once cells reach confluence the culture supernatant are removed and the cells infected with the flavivirus to be tested at a multiplicity of infection (MOI) 5. After 1 hour of infection at between 33-37°C, the virus is removed and the cells washed in culture media and replaced with 1ml of culture media or 1ml 1% agarose in culture media overlay. Drug to be tested is added at various concentrations to the separate wells of flavivirus infected cells. The flavivirus infected cells are incubated at between 33-37°C for 4-12 days or until plaques are present.

The culture supernatant are removed and the cells stained with neutral red or crystal violet stain or the agarose is stained with dye. Plaques are counted and the plaque forming units (PFU) calculated. PFU are compared between wells that had drug added against wells without drug. If the drug inhibits the flavivirus then there is a reduction of plaques present for that well.

Although the invention has been described with reference to certain examples and preferred embodiments, it will be understood by those skilled in the art that variations and modification in keeping with the principles and spirit of the invention described herein are also encompassed.

5

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Miller, C. (1986) Ion channel reconstitution. (ed.) Plenum Press, New York and London.

Sunstrom NA, Premkumar LS, Premkumar A, Ewart G, Cox GB, Gage PW (1996), Ion channels formed by NB, an influenza B virus protein. J Membr Biol. 1996 Mar;150(2):127-32

DATED this 31st Day of May 2004
BALDWIN SHELSTON WATERS
Attorneys for: BIOTRON LIMITED

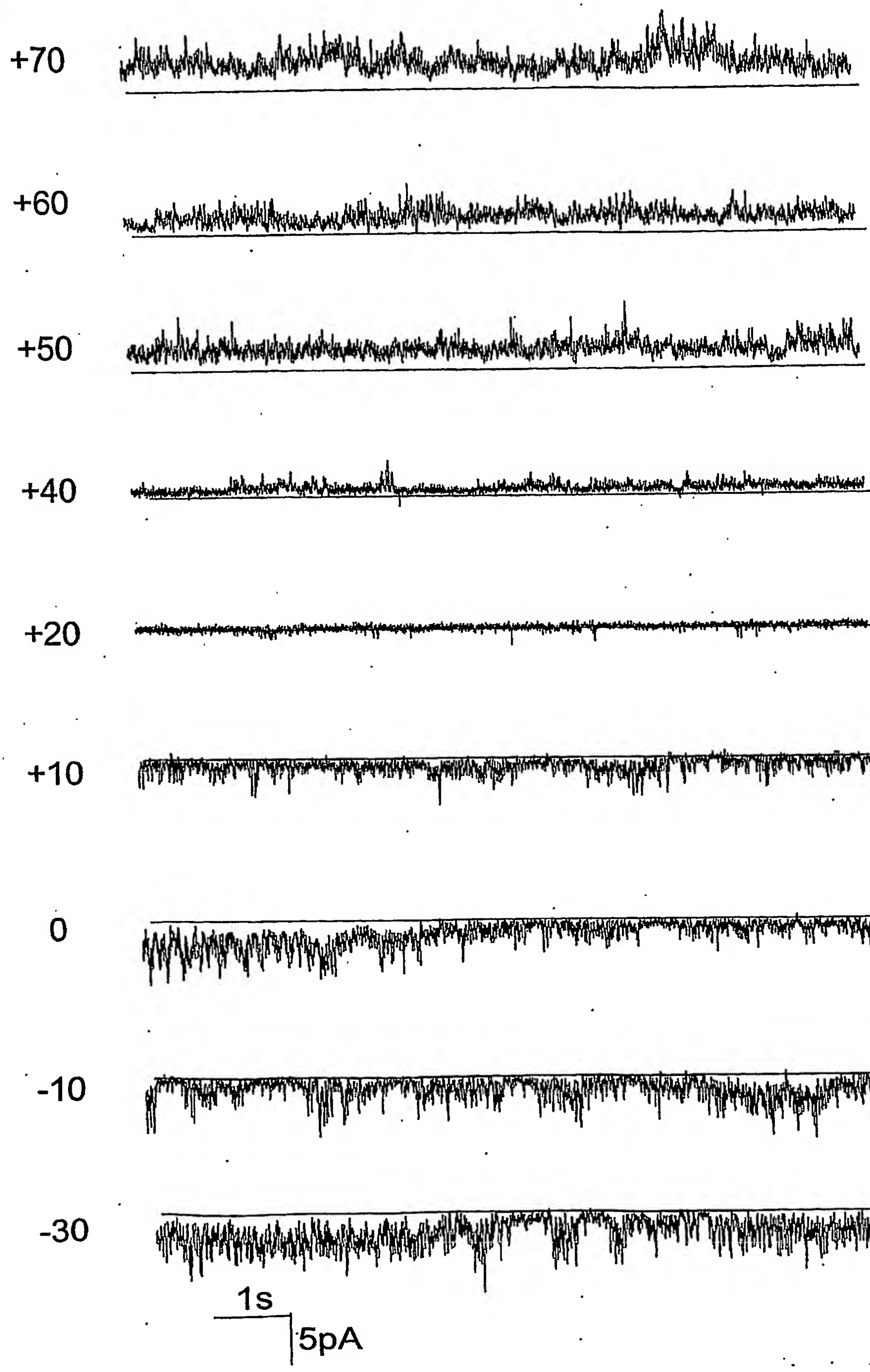

FIGURE 1

Block of dengue virus channels by HMA

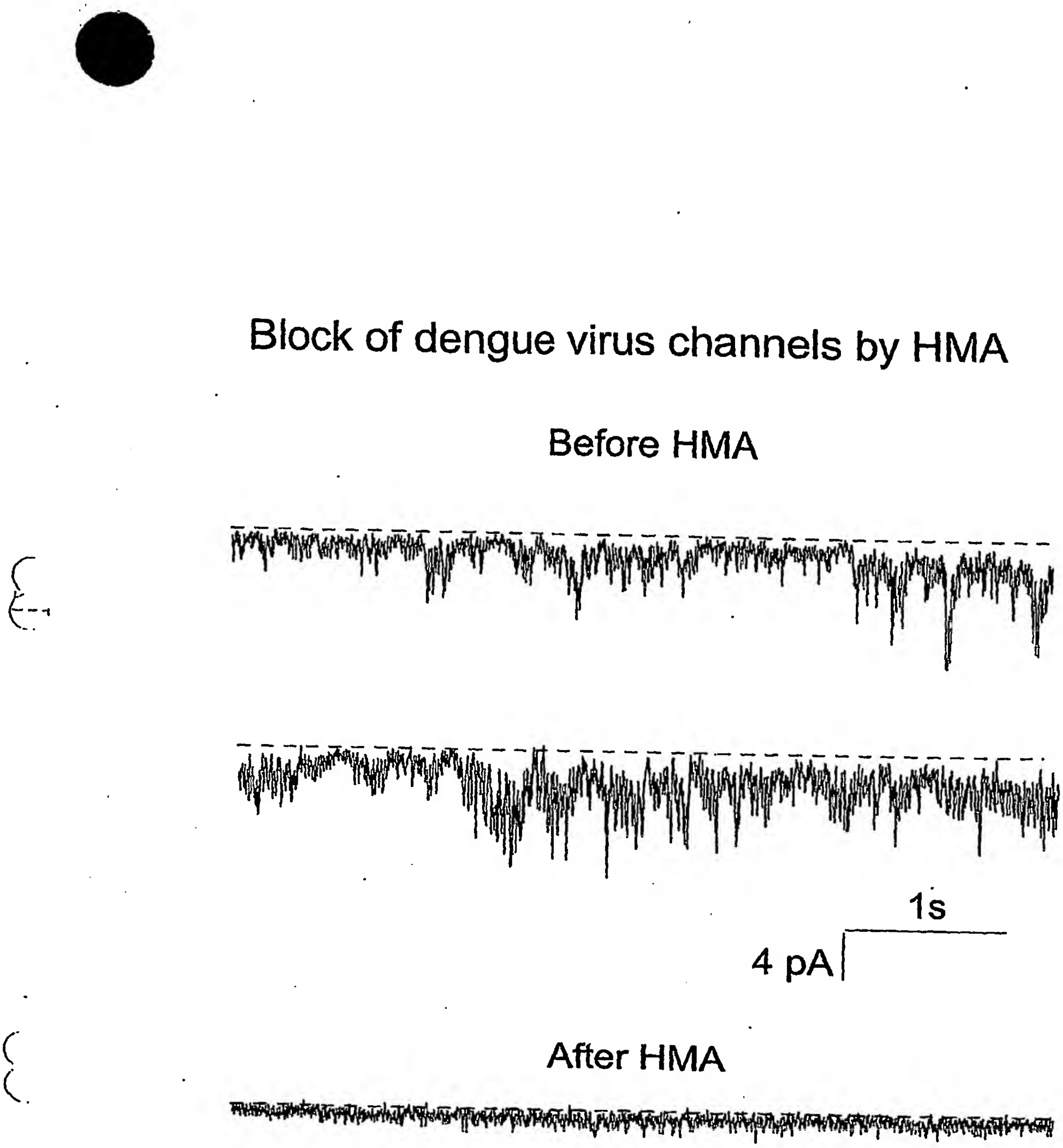

FIGURE 2